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SOME CONSIDERATIONS IN THE USE OF POINT QUADRATS FOR THE ANALYSIS OF VEGETATION

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[*Manuscript received July* 17, 1951]

Summary

The use of vertical pins or point quadrats in the analysis of vegetation, and the statistical treatment of results obtained with them, are discussed on the basis of data collected in different parts of Victoria. Results are expressed in terms of the proportion of the ground covered by each species ("percentage cover"), the average number of layers of foliage covering each point of ground ("cover repetition"), and the proportion of each species in the vegetation as a whole ("percentage of sward").

It is shown that pin diameter affects the results markedly, except those for percentage of sward. Percentage cover and cover repetition both tend to be over-estimated by pins.

Equal distribution of points over the area under study is advocated, rather than random distribution of individual points or groups of points. Where changes in the vegetation are the main subject of interest, successive observations should be made at the same points.

The use of transformations in the statistical treatment of the data is discussed. It is shown that the number of contacts of a pin with a given species can generally be fitted by a negative binomial distribution, if the number of points at which there is no contact be ignored.

Subjective factors may lead to consistent differences between observers recording the same vegetation by the point-quadrat method, but these differences are small compared with those occurring with many other ecological techniques.

I. Introduction

For purposes of analysing vegetation, sample areas or quadrats varying widely in size have been used. The extreme limit of this variation is represented by the point quadrat, virtually without area. If a narrow rod or pin is passed vertically through the vegetation, the plants it touches are those vertically over a single point of ground, and recording these contacts at a large number of points can provide information as to the composition of the vegetation.

This method appears to have originated in New Zealand in 1925, and the first oblique reference to it was published by Cockayne in 1926. From 1927 onwards a number of papers appeared recording results obtained by its use (Levy 1927*a*, 1927*b*; Levy and Smith 1929; Levy and Davies 1929, 1930), but the first description was that of Du Rietz (1932). The following year, Levy (Levy and Madden 1933; Levy 1933) himself published a rather fuller description of the method; a frame supported on two legs contained 10 ver-

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tical holes, 2 in. apart, through which hat pins could pass. The frame was placed at a number of points within the area under study; at first these points were spaced equally (Levy and Smith 1929), but later they were selected at random (Levy 1933; Levy and Madden 1933). In earlier work only a list of species touched by each pin in its descent was recorded, but later the number of contacts with each species was counted. This made possible the analysis of the results in four ways:

(1) "The percentage of ground covered by each species," i.e. the number of points per 100 examined at which contact was made with the species (called "percentage cover" below).

(2) "The percentage cover each species is contributing to the total area." This is the value under (1) divided by the sum of the values under (1) for all species and multiplied by the percentage of ground with any vertical cover.

(3) "The relative frequency of each species in the cover," i.e. the total number of contacts with the species per hundred points examined.

(4) "The percentage each species is contributing to the pasture sward," a value obtained by expressing that under (3) as a percentage of the sum of values under (3) for all species (called "percentage of sward" below).

A point-quadrat method modified from that described by Du Rietz was adopted by Lindquist (1932) for the analysis of the field layer in Swedish beech forests; he used 100 points equally spaced over a square-metre quadrat, the rods being 3.5 mm. in diameter; Böcher (1935), Julin (1948), and Arnborg (1943, 1949) later made use of the same technique. The method as described by Levy and Madden was recommended to British agrostologists by Davies (1931) and Fenton (1933, 1934). In subsequent years the method has been used extensively, particularly in America, for grassland investigations (Hanson 1934, 1950; Tinney, Aamodt, and Ahlgren 1937; Sell 1941; Ellison 1942; Drew 1944); in some cases, the pins were inclined at an angle of 45° instead of vertical (Tinney, Aamodt, and Ahlgren 1937; Henson and Hein 1941; Hein and Henson 1942; Arny and Schmid 1942; Arny 1944; Rhoad and Carr 1945; Sprague and Myers 1945; Musser 1948; Leasure 1949). Point-quadrat methods have also been used in South Africa (West 1937), Finland (Charpentier 1940; Charpentier and Saarela 1941), Argentina (Burkart 1941), and Ceylon (Eden and Bond 1945; Bond 1947). In Australia they have been applied to the analysis of South Australian pasture vegetation (Crocker and Tiver 1946, 1948), and since 1945 in studies of the grasslands of the Victorian Alps (Fawcett and Turner, unpublished data).

Despite the considerable number of investigators who have made use of the point-quadrat method, remarkably little has been done to study the soundness of its bases and details of its application. Levy and Madden (1933) tested the method on cards distributed over an area of 14 sq. ft., and obtained satisfactory agreement between analyses by their methods (1), (2), and (4) and the values derived from direct measurement. Lindquist (1932) tested the method on distributions of pieces of paper cut to three sizes— 50 × 5 mm.,

20 × 20 mm., and 200 × 200 mm.—representing different types of foliage. These pieces were distributed without overlap over an area of 1 sq. m., with deliberate over-dispersion or under-dispersion. He found that the ground covered was consistently over-estimated, but much more with the smaller pieces than with the largest size, and more when the pieces were under- than over-dispersed. For the smallest pieces, when the true percentage cover was 37.5 per cent., the value obtained with over-dispersion was 45.82 per cent., and with under-dispersion 78.52 per cent.

The variance of data obtained by point quadrat analysis has been studied by Clarke, Campbell, and Campbell (1942), Drew (1944), Crocker and Tiver (1948), and Leasure (1949), but the statistical methods used were often inefficient or faulty, and the results inconsistent.

In view of the inadequacy of fundamental knowledge concerning a method of some popularity, with much to commend it from both theoretical and practical viewpoints, a detailed study of certain aspects of it was considered worth while. No comparisons with dry-matter analyses were made; it was considered that point-quadrat analysis should be judged on its own merits as an ecological technique, and that its value was independent of agreement with other ecological techniques which, *from the ecological point of view*, had no greater *prima facie* validity.

Of the methods of analysis proposed by Levy and Madden (1933), only "percentage cover" and "percentage of sward" were considered. The second method, "the percentage cover each species is contributing to the total area," seems to have little significance, and may even be misleading; it expresses nothing that is not better expressed by the percentage of bare ground on the one hand and the figures for "percentage of sward" on the other. Levy's third method ("relative frequency") gives a value largely dependent on that under (1); but if it is divided by (1) one obtains a figure without direct dependence upon (1), which estimates the average number of times the ground is covered by the species in question *where it is present*, and this quantity, termed "cover repetition," has been used in place of Levy's "relative frequency" in the present study.

Modifications of Levy's methods of analysis have been suggested by other writers. West (1937), Clarke, Campbell, and Campbell (1942), and Coupland (1950) recorded contact at ground level only, thus obtaining an estimate of basal area. Hanson and Whitman (1938) and Henson and Hein (1941) appear to have used a modified form of Levy's method (2). Tinney, Aamodt, and Ahlgren (1937) and Drew (1944) recorded only the *first* species with which contact was made at each point; this has the grave disadvantage that the percentage cover by lower-growing species is greatly underestimated, though it might be useful in estimating the degree of dominance by different species. Crocker and Tiver (1948) used a combination of Levy's methods (2) and (4), multiplying the figures for percentage of sward by the fraction of the ground covered by any sort of vegetation; this has the putative advantage of including the area of bare ground in the same analysis with the proportional composi-

tion of the "sward," but unless used with great care such a treatment of the data, involving a combination of quantities different in kind, is likely to lead to misapprehensions, and may cause real differences to be ignored.

In the present investigations, the data were treated, then, in three ways:

(*a*) "Percentage cover," corresponding with Levy's method (1);

(*b*) "Cover repetition"; the mean number of layers of foliage of a species covering the ground vertically; and

(*c*) "Percentage of sward," corresponding with Levy's method (4).

"Percentage cover" and "cover repetition" are concerned with individual species independently; between them, they contain the whole of the information the technique yields in respect of each species considered separately. The figures for "percentage of sward" express the relationships among the species, and though containing no more information than is available in "percentage cover" and "cover repetition" they enable relevant points to be grasped more readily; moreover, the variability of "percentage of sward" cannot be deduced from that for "percentage cover" and "cover repetition." To attempt to express the results of point-quadrat analysis by a single figure for each species, as is done for instance by Crocker and Tiver (1948), is to sacrifice much of the information one has been at such pains to collect.

In subsequent pages the three methods of treating point-quadrat data—"percentage cover," "cover repetition," and "percentage of sward"—are considered *seriatim*, for the problems they involve are in the main distinct.

The observations on which this paper is based were collected in 1949 and 1950 in various parts of Victoria. The majority come from the Bogong High Plains, a plateau at 1700 m. in the north-east of Victoria, covered with alpine grassland, snow gum (*Eucalyptus pauciflora* Sieb.) occupying the more sheltered parts. Others were collected south-east of Melbourne, on the heaths east of Frankston and on sand dunes along the shores of Port Phillip Bay; yet others were obtained on waste ground in the northern Melbourne suburbs and at Blackwood in the Dividing Range, 45 miles north-west of Melbourne. Unless otherwise stated, all results were obtained with pins 4.08 mm. in diameter arranged in frames with places for 10 spaced at 8.9 cm.

II. Estimation of Percentage Cover

In this use of point quadrats, the purpose is to estimate the proportion of ground covered vertically by the species in question. Only presence or absence of each species at each point is recorded, and the results are thus comparable with those for frequency obtained with larger quadrats. It is necessary to consider how the technique should be used to obtain the closest possible agreement of the estimate and the true proportion of ground covered, with a given limited amount of observational work. The most important factor that may be varied to attain this end is the distribution of the point quadrats, but the effects of size of pin and of personal characteristics of the observer were also studied.

(a) Size of Pin

The projection on a horizontal surface of the foliage of most species forms an intricate mosaic; the dimensions of the elements of this mosaic are not greater than those of the leaves, and may be substantially less if many of the leaves are far from horizontal. In broad-leaved plants, the spaces between the projections of the leaves are usually much narrower than these projections

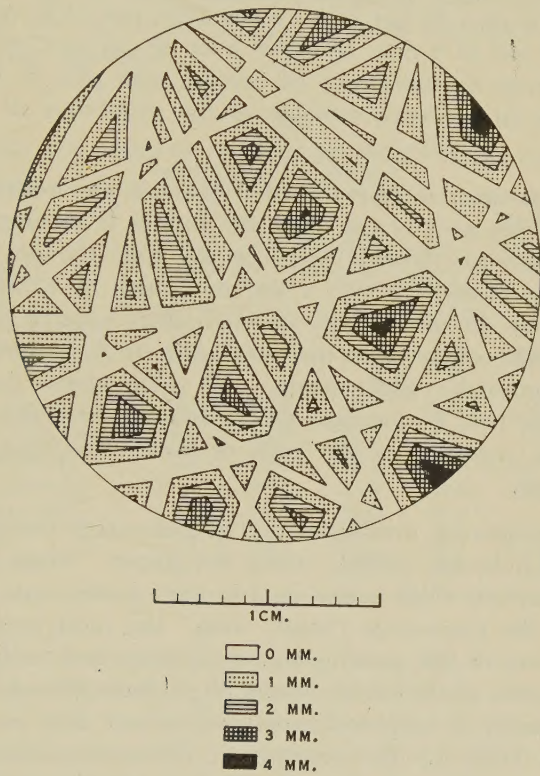

Fig.1.—Projection of part of tussock of *Ammophila arenaria,* showing areas over which contact would be made with the foliage by pins of the diameters stated; each zone is understood as including those with less dense shading.

themselves, for leaves of successive tiers are often arranged so that the lower ones tend to fill the spaces between those above. Under most plants, the ground not covered vertically by foliage is thus distributed in many small areas, at least one dimension of which is usually not more than a centimetre or two. These areas in sum may represent a large part of the whole; but the possibilities of systematic error in their measurement through faulty outlining are very considerable. Systematic errors of this sort occur when frequency of contact with a vertical pin is used as a method of measuring their area. Assuming that the vegetation is still and the pin moves vertically through it, the area

within a single open space in which the centre of the pin must fall if the pin is not to touch the foliage is less than the total area of the open space by an amount depending on the diameter of the pin. This effect is shown in Figure 1, which represents the projection on a horizontal plane of part of a tussock of *Ammophila arenaria.* The true projection occupies 51 per cent. of the area of the circle. A pin of 1.0 mm. diameter—which would hardly have adequate rigidity for practical use in most types of vegetation—would make contact with plants over 82 per cent. of the area, one of 2 mm. diameter over 96 per cent., and one of 3 mm. diameter over 99 per cent. of the area of the whole circle. Hence the frequency of contact with pins of finite diameter is bound to provide an excessive estimate of the proportion of ground covered by a species.

This source of error is accentuated if there is any relative movement of the projections of pin and foliage while the former is moving downwards. If this occurs, for instance through wind, by the time the pin has reached the ground, it is often in contact with a leaf or branch of which the position of rest does not lie on its direct path—through their relative displacement, this leaf or branch made contact with the pin during its downward movement, and remains in contact under slight strain. All such relative lateral movements of pin and foliage tend to increase the probability of contact between them above that to be expected on the basis of their projections on a horizontal plane while at rest.

As already mentioned, over-estimation of percentage cover by pins was demonstrated by Lindquist (1932), using cut paper. West (1937) surmised that "the comparatively thick points used in our extemporised apparatus would tend to magnify the Percentage (basal) Area," but most workers do not seem to have been aware of this possible source of error, and so far as I am aware no tests *in vegetation* of the effects of size of pin have hitherto been performed. Accordingly, a series of such trials was carried out, and some of the results are presented in Table 1. For comparison, observations were made with an apparatus consisting of a tube hung in gimbals with cross-wires at each end. Observations of the vegetation through this tube enabled one to determine whether the ground vertically beneath the centre line of the tube was bare, or was covered by some particular species, and such observations could be regarded as records obtained with a dimensionless optical point quadrat; these are the frequencies shown in the zero diameter column. All the data in this table were obtained from points distributed at random in quadrats 1 m. square. The significance of differences between frequencies obtained with the three types of point quadrat was determined by the χ^2 test.

In Table 2, the frequencies of contact with the more abundant species (all those for which the mean exceeded 5 per cent.) in much larger areas of alpine pasture on the Bogong High Plains are given, as determined with pins of two sizes. The pins were used in frames, each holding 10, placed at each of 200 randomly selected points (100 for the narrower pins) in each of the two

areas. The significance indicated in the last column is based on a t test using the variance between frame means after angular transformation (see below).

In all the data recorded in Table 1, the cross-wire apparatus gave a lower frequency of contact than the smaller pins, many of the differences being highly significant. The differences between the two types of pin were not so marked, though in three instances the narrower pin gave a significantly lower frequency. In the more extensive series of observations in Table 2, in all cases but one the narrower pin gave a lower frequency and in many the difference is highly significant.

TABLE 1

FREQUENCY (PER CENT.) OF CONTACT BETWEEN FOLIAGE AND PINS OF DIFFERENT DIAMETERS

Locality	Species	No. of Points	Pin Diameter (mm.)			Significance (P) for Difference in Pin Diameter	
			0	1.84	4.75	0-1.84 mm.	1.84-4.75 mm.
Seaford	*Ammophila arenaria*	200	39.0	66.5	71.0	< 0.001	> 0.05
	Ammophila arenaria	200	60.5	74.0	82.0	0.001-0.01	> 0.05
Black Rock	*Ehrharta erecta*	200	74.5	87.0	93.5	0.001-0.01	0.01-0.05
Sorrento	*Lepidosperma concavum*	200	19.5	22.0	27.5	> 0.05	> 0.05
	Spinifex hirsutus	200	35.0	48.5	61.0	0.001-0.01	0.01-0.05
Carlton	*Fumaria officinalis*	200	20.5	31.5	30.0	0.01-0.05	> 0.05
	Ehrharta longiflora		24.5	25.5	37.5	> 0.05	0.01-0.05
	No contact		53.0	42.5	38.5	0.01-0.05	> 0.05
	Lolium perenne	200	65.0	85.5	82.5	< 0.001	> 0.05

Since the error in estimating percentage cover by the use of pins depends in part on the size distribution of gaps in the foliage, it will in general be greater for species with smaller leaves, as Lindquist (1932) concluded. This implies that a constant correction factor for pins of a given size is out of the question. Within a single species, environmental conditions affecting the size of leaves and the habit of the plant may cause the error in estimating percentage cover to vary, but these variations will in general be small, and a correction factor or curve could probably be used. At high values of percenage cover for microphyllous species, broad pins will, however, be an insensitive means of estimation, and there will be a range where the true percentage cover falls short of 100 yet all the pins make contact.

(*b*) *Distribution of Points*

The next question to be considered is how points of observation should be distributed in the area of vegetation under study. In most work of this type, the pins have been spaced evenly along a frame, commonly containing 10, and the frames have been located and oriented at random within the area. The desirability of placing the points in close groups, as against their distri-

bution as individual observations, must be considered, as also the wisdom of arranging these groups or individual observations at random.

Since, in most types of vegetation, the scale of the pattern presented—the average distance between individuals or groups of individuals of the same species—is considerably greater than the size of the frame used for placing the pins (1 m. or less in length), it seems probable, as suggested by Blackman (1935), that the probability of hitting a given species will vary less between

TABLE 2

FREQUENCY (PER CENT.) OF CONTACT BETWEEN FOLIAGE AND PINS OF DIFFERENT DIAMETERS, BOGONG HIGH PLAINS

	Species	Pin Diameter (mm.) 2.05	Pin Diameter (mm.) 4.08	Significance (P) for Difference in Pin Diameter
Plot *A* (298 sq. m.)	*Carex hebes*	64.1	76.55	< 0.001
	Poa caespitosa	5.7	8.15	> 0.05
	Rumex acetosella	6.1	11.15	0.001-0.01
	Viola betonicifolia	9.6	14.60	0.001-0.01
	No contact	20.9	7.60	< 0.001
Plot *B* (670 sq. m.)	*Carex hebes*	57.1	70.20	0.001-0.01
	Poa caespitosa	15.7	13.70	> 0.05
	Asperula gunnii	5.0	6.45	> 0.05
	Viola betonicifolia	4.5	6.60	> 0.05
	Microseris scapigera	7.2	8.00	> 0.05
	No contact	14.1	6.95	< 0.001

the different sections of one frame than between different positions of the frame. This is illustrated in Table 3, the data for which come from Plot *A* of Table 2. The binomial distribution of frequencies in samples of 10 has been computed for the overall mean frequency of contact, and this has been compared with the actual frequencies observed in the groups of 10 provided by the 200 frame locations. It will be seen that the spread of values is much greater than would be expected on the assumption that the 10 pins in each frame represented random samples of a single population. With some of the scattered shrubs on this plot, the discrepancy would be even greater; for instance, *Phebalium podocarpoides* occurred in only two frames, but in these four and three pins respectively made contact with it; and *Grevillea australis* occurred in four frames only, in one of which seven pins touched it. Thus it is clear that the proportion of ground covered by each species differs from frame to frame, or in other words that the variation in percentage cover within the frame is less than that between frames.

A small part of the variation between frames may be ascribed to personal differences among observers, for the frames were recorded by three different teams, each consisting of three workers who observed in rotation. It is shown later that such personal differences are not altogether negligible, but they are much smaller than those recorded here between frames.

From this fact that the variation in percentage cover within the frames is less than that between frames, it may be deduced that a given number of points distributed individually over the plot would give a more precise estimate of mean percentage cover than the same number grouped in frames. Conversely, if the precision attained by use of 2000 points arranged in 200 randomly distributed frames is satisfactory, equal precision would be possible by use of a smaller number of individual points. Since the variance of the estimate of percentage cover obtained in this way would, after angular transformation (see below), be given by the expression $8100/\pi^2 n$, where n is the number of points, one may readily calculate the number of individual points that, distributed at random, would give an estimate with the same variance as that observed for points arranged in frames. These results are given in Table 4, for the same area as figures in Table 3.

TABLE 3

FREQUENCY OF CONTACT WITH PINS IN 200 FRAMES OF 10 PINS, COMPARED WITH EXPECTATION ON A BINOMIAL DISTRIBUTION

	Number of Frames in which Stated Number of Pins made Contact With									
	Carex hebes		*Poa caespitosa*		*Rumex acetosella*		*Viola betonicifolia*		*Asperula gunnii*	
No. of Pins Making Contact	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
0	0	0	115	85.5	95	56.0	60	42.2	176	142.2
1	3	0	41	75.8	43	69.3	56	71.2	6	49.3
2	3	0	22	30.3	31	38.6	42	53.8	6	7.7
3	3	0.4	14	7.2	16	12.7	28	24.1	4	0.7
4	3	2.5	4	1.1	11	2.8	9	7.1	4	0.1
5	14	9.4	4	0.1	2	0.5	2	1.4	3	0
6	25	25.5	0	0	1	0.1	3	0.2	1	0
7	35	47.7	0	0	1	0	0	0	0	0
8	33	58.4	0	0	0	0	0	0	0	0
9	38	42.3	0	0	0	0	0	0	0	0
10	43	13.8	0	0	0	0	0	0	0	0
χ^2	91.9		50.58		52.39		17.27		56.68	
n	4		2		2		3		1	
P	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	

An alternative approach to the question of the relative efficiency of frames and individual points in estimating percentage cover may be illustrated with *Grevillea australis*. This plant occurred in four frames only of the 200 recorded on the plot, but in these four frames a total of 15 pins (out of 40) made contact with it. These results enable one to estimate the probability that a frame will include a record of *Grevillea* as 0.02, and the probability, within such a frame, that any particular pin will make contact with it as 0.375. Given these

probabilities, it is possible on binomial theory to compute the distribution of numbers of pins making contact in samples of 200 frames, and hence to determine the variance of these numbers, which is found to be 60.8. Now, for samples consisting of individual points distributed at random, the variance of the number of pins making contact with *Grevillea* will be $kp\,(1-p)$, where k is the number of points, and p the probability of contact (i.e. $0.02 \times 0.375 = 0.0075$). Thus the number of individual points required to give a variance of the estimate of percentage cover equal to that for 2000 points arranged in frames may readily be computed to be 490. Admittedly, this treatment assumes that, within frames where the species occurs, the numbers of pins making contact with it are distributed binomially, and in other species this is known not to be true (see Table 3—data for *Carex hebes*); but the deviations from this assumption that have been observed have had the effect of increasing the range of variation, and their effect if taken into account in the calculations above would thus be to increase the variance of the estimate derived from frames, and hence to increase the relative precision of the estimate by individual points.

TABLE 4

NO. OF INDIVIDUAL POINTS REQUIRED TO GIVE ESTIMATES OF PERCENTAGE COVER OF EQUAL PRECISION TO THOSE OBTAINED FROM 2000 POINTS IN FRAMES OF 10

Carex hebes	568
Poa caespitosa	902
Rumex acetosella	771
Viola betonicifolia	866

As a final test of the relative efficiency of sampling the vegetation by individual points and by frames, both methods were used on Plot *B* of Table 2. In addition to the observations at 2000 points in frames, individual pins were distributed at random at 1000 points over the plot, and their contacts were recorded. The frames or points, as the case might be, were then randomly assorted into groups, each containing records from 100 pins, and the variance among the percentage cover estimates from these groups was computed after angular transformation. The results are shown in Table 5.

The variances do not differ significantly as between species, but the difference between the two methods is highly significant, and indicates that, taking all five species together, 670 individual points would be required to give a precision equal to that obtained with a sample of 2000 points in frames of 10.

Apart from the relative value of individual points and groups of points such as are provided by the frames, the question must be asked whether the points or groups of points should be distributed at random, or whether some

restricted or regular arrangement is desirable. Let us suppose that the problem to be solved is whether the percentage cover by a given species is similar in two plots or not. Even if the true total area covered by the species in each plot could be determined accurately, without any observational error, these errorless estimates of the two means would not in themselves be enough to provide a basis for useful comparison of the two plots; without estimates of variability, such accurate data for percentage cover would be valueless. Besides the errors in estimation, which in the present case we have by hypothesis excluded, there is also the large variation in the composition of the vegetation from place to place in the plot. Though the percentage cover were greater in Plot *A* than in Plot *B*, it might well happen that the relation would be reversed if the area of one of the plots were slightly increased or decreased to include a few more or a few less plants of the species in question. Clearly a difference which thus depended on the fortuitous location of the plot boundaries would be without any fundamental significance, and a criterion is needed to enable one safely to ignore such chance differences, and to identify those representing a real and consistent difference in the vegetation of the two plots. Thus a satisfactory distribution of points for observation will not only give unbiased estimates of the mean percentage cover within the plot, but will also give estimates of the variability of this value over the plot. At the same time, the sampling error of the percentage cover estimates should be minimal.

TABLE 5

VARIANCE OF PERCENTAGE COVER ESTIMATES FROM SAMPLES OF 100 POINTS (ANGULAR TRANSFORMATION)

Species	10 Frames Each with 10 Points	100 Individual Points
Carex hebes	35.03	10.92
Poa caespitosa	35.06	9.54
Viola betonicifolia	16.57	16.48
Asperula gunnii	37.83	8.23
Microseris scapigera	21.12	3.61

The importance of variation in percentage cover over an area has already been indicated by the smaller variability of points within frames than that between different frames (see Table 3 and accompanying text). That it also occurs on a larger scale, even where not immediately apparent to the eye, may be made clear by some data from another area (of 448 sq. m.) on the Bogong High Plains. In 1949 this land, originally selected for uniformity of vegetation, was divided into five strips of equal area, each of which was sampled by 20 frames each containing 10 pins. Some of the results are shown in Table 6. For each species, an analysis of variance was computed, after angular transformation (see below), and the variance ratios quoted are the ratios of the variance between strip means to that between frames within strips. Though

the differences in the less abundant species are not significant (both are shrubs, and hence the variance between frames is high), the differences in the proportion of bare ground almost reach significance, and those for the dominant, *Poa caespitosa,* are very highly significant.

TABLE 6

PERCENTAGE COVER BY CERTAIN SPECIES ON FIVE ADJACENT STRIPS OF LAND

Strip	*Poa caespitosa*	*Hovea longifolia*	*Phebalium podocarpoides*	Bare Ground
1	61.5	15.5	14.5	9.5
2	81.0	15.5	10.0	1.0
3	88.5	5.5	10.0	2.0
4	76.0	8.5	14.0	8.0
5	54.0	17.5	14.0	10.5
Variance ratio, between strips/within strips, after angular transformation	47.81	1.69	0.34	2.29
P	< 0.001	> 0.05	> 0.05	> 0.05

The reasons in plant ecology for wishing to separate variance due to causes operating on different scales are not the same as the considerations involved in agricultural experimentation, where differences with location within an experimental area are undesirable complications, and are measured only incidentally in the course of attempts to restrict their influence on the experimental results. In the common "randomized block" type of design, the area is divided into a number of blocks, each further divided into as many plots as there are treatments, the treatments being allotted at random to the plots within each block. In this way it is possible to prevent differences between blocks affecting the comparisons between treatments, and only that smaller part of the variation in site factors which is operating *within* blocks will affect the treatment differences. The variance between blocks is eliminated with the loss of few degrees of freedom, and the bulk of the degrees of freedom among plots are retained for the study of the treatment differences.

In ecological studies of the differences in vegetation between areas, variation between blocks would be *more* relevant to the comparison than variation within blocks—because it includes all sources of variation within the area, and not merely those operating over short distances only. Hence any degrees of freedom for differences within blocks will be lost to the primary purpose of comparison of the main areas, and the greatest efficiency in this comparison will be reached if all degrees of freedom relate to comparisons *between* blocks — that is, if there are as many blocks as there are points of observation.* Random distribution of replicate points within any area or sub-

* Moreover, this procedure ensures that equal weight is given to all parts of the area, whereas random distribution of points implies that weights given to different portions are themselves allowed to vary at random.

area under consideration will mean that such points contribute no information about the variation of percentage cover over the area, but merely reduce the sampling error of the estimate. To take an extreme case, consider two areas in which half the surface is painted black and half white, but in the one each colour is in a compact block, while in the other the colours are distributed in a mosaic of minute alternating squares. Points placed at random will give on the average estimates of 50 per cent. black on both areas, and sample means will vary equally about this figure; from the sampling results, it will be impossible to distinguish between these two very diverse cases. If, however, the area is divided into small plots for sampling, and a few points are selected at random within each plot, the variance between plot means will be much greater in the first area than in the second (unless the plot size is as small as the mosaic squares of the latter), thus illustrating its greater uniformity. The variance between the means for replicate samples taken in the same way, plot by plot, will also be less than with random sampling.

The increase in precision through restricting the randomization of points for observation each within a separate small plot may be illustrated from a plan drawn by Davies (1945) showing the distribution of species in 1 sq. m. of a Molinietum in a Welsh bog. Seven per cent. of the area is bare peat, and this quantity would be estimated by randomly distributed samples of 100 points with a variance of 6.52. If, however, the area is divided into decimetre squares, and sampled by groups of 100 points, one placed at random within each of these squares, the variance of the estimates so obtained is 5.63.

Taken as individual contributors to mean and variance, point observations under restricted randomization provide no more information about the distribution of species over the area than if randomized freely. It is only when the location of the points in the area and their relation to adjacent points is considered that this information emerges. But the variation among plots will often be non-homogeneous—the differences between adjacent plots will be less than between those more separated—and a question then arises as to what elements in the non-homogeneous variation are appropriate for use as estimates of error in comparisons between different areas. This question is too large to discuss here—it is hoped to deal with it in a subsequent paper.

If point samples need to be taken in each segment of an area, it would often be more convenient to take them at equal intervals along each dimension of the area—a practice analogous with that proposed for yield sampling in agriculture or forestry by Yates (1946)—than to place them at random within each plot. This has, however, the theoretical drawback that the sample may be biased and unrepresentative if the vegetation varies regularly over the area, with a period equal to a multiple or submultiple of the distance between points. This is perhaps rather a remote contingency, but unless there are serious difficulties in random sampling within each plot it would appear that this is slightly to be preferred. For most purposes, sampling error would be irrelevant, and information on it would not be available if one point only

were placed at random within each plot; if an estimate of sampling error were required it would be necessary to place at least two points at random within each plot.

A problem to which point-quadrat methods may often be applied is the determination of changes taking place in vegetation—for instance, the change in botanical composition of a pasture under grazing, the course of loss in vegetational cover under soil erosion, or the gradual deterioration in cover during a prolonged drought. For this purpose, assessment of the condition of the vegetation at any one time has no direct relevance, and the quantities whose estimation is required are the *changes* in percentage cover by each species from time to time, and the variations in those changes over the area studied. If successive random samples are taken, whether the randomization is free or restricted as suggested above, the error variance of the estimate of change will of course be double that for the mean of a single sample, and will be less if the randomization is restricted within plots than if it is free. Variation over the area in the changes will be superimposed upon this error variance if the area has been sampled by plots, and the distribution of the differences between successive observations on the same plot may be used to indicate the consistency of the changes over the area.

However, a much more powerful means than division into plots is available for reducing the error of estimates of changes, namely, to take successive observations at the *same* series of points. This will enable sampling errors to be excluded from the estimate of change, except for those arising from movement of the foliage and in so far as the points chosen are not completely representative of changes occurring in the area as a whole. The differences observed over a series of fixed points chosen by free or restricted randomization will give an unbiased estimate of the changes in the area as a whole during any one period. But changes over successive periods at a given point are likely to be correlated, and hence caution must be used if the observations are intended to show changes over several successive periods; estimates of these changes will not be independent, and if the estimate of increase in percentage bare ground (for instance) over the first period exceeds by chance the true mean for the whole area, then this deviation is likely to occur again in the second period. This difficulty may be overcome by using a different series of points for estimating the change over each period. For instance, if the practice advocated above of dividing the area into small plots within each of which a single point is selected be adopted, an observation would be made in Plot *A* at point 1 on the first occasion, points 1 and 2 on the second, and 2 and 3 on the third, and so forth. Thus the estimate of change at point 1 during the first period would be independent of that at point 2 during the second period —apart from the consistency of changes in the same plot, a point of interest that could be elucidated by analysis of variance—and the mean estimates of change over the whole area would likewise be independent in successive periods.

The great superiority of successive observations at fixed points (comparable with permanent quadrats) over successive series of independently ran-

domized points is shown by the results recorded in Table 7. These were obtained from another area in the Bogong High Plains, at the head of Pretty Valley. This area was 74 m. long, and on average 12 m. wide. In 1949, 100 frames of 10 points distributed at random were recorded, and in 1950 200 similar frames; further observations were made on 121 frames placed at the same points (within the limits of measurement—say, 5 cm.) in each of the two years, these points being equally spaced at intervals of 1.83 m. across the plot and 3.30 m. along it. The variances quoted in the table are expressed on a basis comparable between the two methods—that is, for observations on one frame in each year. In all cases but one, the variance is much greater with randomly distributed points than with fixed points.

TABLE 7

CHANGES IN PERCENTAGE COVER AS ESTIMATED BY RANDOM POINT QUADRATS AND PERMANENT POINT QUADRATS

	Permanent Point Quadrats			Random Point Quadrats		
Species	Mean, 1949	Mean, 1950	Variance of Differences	Mean, 1949	Mean, 1950	Variance of Differences
Poa caespitosa ("fine grass")	54.6	53.4	336.8	49.9	54.7	1456.1
Poa caespitosa ("horny grass")	12.0	15.3	169.8	15.4	14.0	871.5
Leptorhynchus squamatus	19.3	18.1	312.0	18.8	19.1	870.5
Carex breviculmis	18.3	18.5	346.1	20.7	15.6	406.5
Celmisia longifolia	6.4	8.1	134.5	4.1	7.3	239.6
Scleranthus biflorus	2.2	2.1	29.2	1.2	2.5	58.4
Ranunculus lappaceus	3.2	3.9	78.6	2.3	3.0	67.1
Danthonia semiannularis	3.9	3.1	82.6	3.9	4.2	143.2
Bare ground	3.2	2.4	31.0	2.1	3.5	208.4

(c) *Statistical Treatment of Results*

In common with other variables in which a part is expressed as a proportion or percentage of the whole, estimates of percentage cover from samples will not be distributed normally about their mean with a variance independent of the mean. Their distribution, however, is such that, for large samples, the function

$$\text{arc sin} \sqrt{\frac{\text{percentage cover}}{100}}$$

is distributed approximately normally, and (measured in degrees) has a variance $8100/\pi^2 n$ (n being the number of observations in the sample) independent of the value of the mean (Fisher and Yates 1938). The equalization of variances by this angular transformation is demonstrated by the results in Table 8. These observations were made with the cross-wires instrument already described, on

areas of 1 sq. decimetre. In each such area, 10 series of 100 observations were taken, and the variances of the percentage cover estimates so obtained were determined; for each species five or six areas were studied in which its abundance varied widely.

TABLE 8

VARIANCE OF PERCENTAGE COVER ESTIMATES DERIVED FROM RANDOM SAMPLES OF 100 POINTS

Species		Area A	B	C	D	E	F
Leucopogon parviflorus at Seaford	Mean	96.8	48.9	36.2	8.1	0.7	—
	Variance	4.84	21.88	9.07	9.07	1.12	—
	Variance after transformation	17.21	7.18	3.12	3.12	15.95	—
Ehrharta longiflora at Carlton	Mean	97.6	85.9	57.1	6.8	2.1	—
	Variance	2.04	29.88	33.43	5.51	2.77	—
	Variance after transformation	13.21	20.16	11.27	8.32	19.61	—
Pteridium aquilinum at Blackwood	Mean	99.2	83.5	46.2	37.2	2.5	—
	Variance	0.84	13.39	40.62	68.62	1.83	—
	Variance after transformation	14.93	8.25	17.64	25.03	7.18	—
Hypochoeris radicata at Blackwood	Mean	67.7	44.9	18.4	7.4	1.6	0.3
	Variance	18.01	13.66	7.38	1.82	1.82	0.23
	Variance after transformation	7.44	8.30	4.16	2.20	19.11	7.58

It will be seen that the variances, differing markedly with the mean in the original percentage data, become much more uniform within a species after angular transformation; in each of the four species, the differences among the transformed variances are non-significant, as judged by the Bartlett test. It is surprising that this uniformity does not extend to interspecific comparisons ($\chi^2 = 9.09$; $n = 3$; P nearly equal to 0.03), as might be expected; the mean variances for *Ehrharta longiflora* and *Pteridium aquilinum* are moreover found to differ significantly from the variance (8.21) expected between percentages based on 100 observations after angular transformation. These discrepancies may perhaps be due to imperfect dimensionlessness of the point quadrat as determined by cross-wires and eye, or to movements of foliage in the wind affecting results with some species more than others.

This transformation is not always needed for work with percentage cover data. For direct comparison of means, as in Table 7 for instance, the means of large samples have a sampling variance whose distribution approaches normality, and provided the magnitude of the means under comparison does not differ widely the differences in their variance need not be a source of difficulty. Where, however, one wishes to include in the same treatment samples of which the means differed widely—if, for instance, one wished to

find whether grazing had the same effect on a given species irrespective of whether it was a major or minor constituent of the vegetation—transformation would be necessary in order to render the variances uniform.

(*d*) *The Personal Factor*

The personal error in determining whether or not a pin touches a given species—that is, in estimating percentage cover—was tested by comparing results obtained by three experienced observers working on waste ground in Carlton. Each observer recorded contacts with the same pins in succession, the pins remaining in position and great care being taken to avoid disturbing the vegetation. To eliminate possible effects of such disturbance, the order in which the observers recorded was varied from pin to pin. In all, 300 points were recorded in this way. An analysis of variance was performed on the data for each species, its presence at a point being treated as unity, its absence as zero. The significant differences shown in Table 9 below are derived from the interaction term (observers × points) in these analyses of variance. Observers *B* and *C* agreed satisfactorily throughout, but observer *A* exceeded their estimates significantly for two species, and fell below theirs for another.

TABLE 9

THE PERSONAL FACTOR IN RECORDS OF PERCENTAGE COVER

Species	Percentage Cover as Estimated by Observer			Significant Difference Between Observers
	A	*B*	*C*	
Lolium perenne	65.3	61.7	66.3	n.s.
Plantago lanceolata	51.3	51.0	51.3	n.s.
Bromus spp.	30.7	23.3	25.0	4.3
Cryptostemma calendulaceum	20.3	23.3	23.0	2.4
Romulea bulbocodium	9.0	7.0	5.3	2.4
Medicago arabica	5.3	6.0	6.0	n.s.
Medicago denticulata	5.3	4.0	4.3	n.s.

Another series of observations was made without moving the frames, each observer placing the pins afresh before recording contacts, in order to test the possible extra source of personal error involved in guiding the pins through the holes in the frame. However, the movement of the pins introduced additional random variance, obscuring even the personal errors in observation noted above, and none of the differences between observers was significant.

Few investigators have studied observer differences in point quadrat work. Ellison (1942) found marked variation in estimates of percentage cover on one of the three areas he studied. The observations were made by using a frame in a fixed position, the pin being dropped afresh for each record, and the results obtained by the different observers for percentage cover by *Buchloë dactyloides* (the only species recorded) ranged from 68 to 87 per cent.; it would seem from the results above that with observers well accustomed to the method considerably better agreement than this can be expected. It may be

mentioned that in the tests above the conditions of observation were more difficult than usual, on account of the need to avoid disturbing the vegetation, and this is more likely to have increased than diminished the variance between observers.

III. Estimation of Cover Repetition

At a point where a species is present, its foliage may cover the ground once, or many times over. The average value of this quantity is here termed "cover repetition," and it is estimated by the number of times each pin hits the species while moving downward through the vegetation.*

(a) Size of Pin

As in the estimation of percentage cover, so here the results are also affected by the diameter of pin used. All the considerations leading to an excessive estimate of percentage cover with a broad pin also tend to increase the number of times it comes into contact with the same species at any one point. Furthermore, if an upper leaf or branch is strained slightly out of its position of rest by the passage of the pin past it, this may affect the position of leaves or branches at a lower level and cause them to make contact where in their position of rest they would not have done so, or *vice versa.* This will tend to increase the variance of the number of contacts.

Table 10

COVER REPETITION BY FOLIAGE OF DIFFERENT SPECIES AS ESTIMATED BY PINS OF DIFFERENT DIAMETERS

Locality	Species	Pin Diameter (mm.) 1.84 Cover Repetition	1.84 No. of Points	4.75 Cover Repetition	4.75 No. of Points	Significance (P) for Difference in Pin Diameter
Seaford	*Ammophila arenaria*	2.03	133	2.09	142	> 0.05
	Ammophila arenaria	2.57	148	2.63	164	> 0.05
Black Rock	*Ehrharta erecta*	2.64	174	3.01	187	0.001-0.01
Sorrento	*Lepidosperma concavum*	1.20	44	1.20	55	> 0.05
	Spinifex hirsutus	1.64	97	1.80	122	> 0.05
Carlton	*Fumaria officinalis*	1.89	63	2.42	60	0.01-0.05
	Ehrharta longiflora	1.27	51	1.67	75	0.01-0.05
	Lolium perenne	2.87	171	3.25	165	> 0.05

Tables 10 and 11 give results for cover repetition similar to those in Tables 1 and 2 for percentage cover. The significance of the differences shown in Table 10 was examined by the χ^2 test applied to the original distribution of numbers of contacts per point; since the observations in Table 11 were made

* The use of the cross-wire apparatus for determining this quantity is hardly practicable, for its use would necessitate moving each upper storey of foliage aside in order to get a clear view of the lower storeys.

by frames, the significance indicated in the last column is based on the weighted variance between frames, the weights being proportional to the number of pins in the frame making contact with the species in question (see below). It will be seen that in most cases the narrow pin gives a lower value for cover repetition than the broad pin, and that often this difference attains a high level of significance. It may be supposed that the cross-wire apparatus, had its use for this purpose been practicable, would have given still lower results.

TABLE 11

COVER REPETITION BY FOLIAGE OF DIFFERENT SPECIES ON BOGONG HIGH PLAINS, AS ESTIMATED BY PINS OF DIFFERENT DIAMETERS, ARRANGED IN FRAMES OF 10

	Species	Pin Diameter 2.05 Cover Repetition	Pin Diameter 2.05 No. of Frames	Pin Diameter 4.08 Cover Repetition	Pin Diameter 4.08 No. of Frames	Significance (P) for Difference in Pin Diameter
Plot *A*	*Carex hebes*	2.20	97	2.93	200	0.001-0.01
	Poa caespitosa	1.68	56	2.53	86	0.001-0.01
	Asperula gunnii	2.73	40	2.29	23	> 0.05
	Viola betonicifolia	1.15	91	1.37	139	0.001-0.01
	Rumex acetosella	1.41	59	1.58	106	> 0.05
Plot *B*	*Carex hebes*	2.58	94	3.32	196	0.01-0.05
	Poa caespitosa	1.95	66	2.04	125	> 0.05
	Asperula gunnii	3.45	24	2.94	49	> 0.05
	Viola betonicifolia	1.15	31	1.47	78	0.01-0.05
	Microseris scapigera	1.41	22	1.59	56	> 0.05

(*b*) *Distribution of Points*

Where the foliage of individuals of a species does not overlap, the cover repetition will depend only on the habit of the plants and their height. Hence it is to be expected that, provided the areas sampled are large enough for few of the individuals present to be cut by the boundaries, the variability of cover repetition data should be small.

In the areas mentioned in connection with Tables 2 and 11, the variation in mean cover repetition between frames has been tested for a number of species, and has been found to be significantly greater than that within frames only* in *Carex hebes,* the dominant, as shown in Table 12; in other words, for this one species differences in cover repetition did in fact occur over the plot, but for other species they were negligible. In another plot on the Bogong High Plains—that at the head of Pretty Valley (see Table 7)—it is also true that the cover repetition by the dominant (*Poa caespitosa* var. "fine grass") varies significantly from frame to frame. This plant forms tussocks, and where the number of contacts exceeded 10 (which often occurred near the centre of

* Apart from *Asperula gunnii* in one of the four series of observations.

a tussock) counting was impracticable. It was therefore necessary to treat these data in two stages; first it was shown that the proportion of points at which the contacts exceeded 10, among those for which the species was recorded at all, varied significantly between frames, the variance being 1979 (with 116 degrees of freedom) after angular transformation, as compared with the theoretical value of 820.7 had there been no differences among the frames. Then differences were tested among the numbers of contacts in different frames for points with no more than 10 contacts, with the results in Table 13. Thus on both criteria *Poa caespitosa* showed significant variation in cover repetition within the plot.

TABLE 12

ANALYSIS OF VARIANCE OF COVER REPETITION BY *CAREX HEBES*, BOGONG HIGH PLAINS (SQUARE ROOT TRANSFORMATION)

Plot	Pin Diameter (mm.)	Between Frames Variance	Between Frames D.F.	Within Frames Variance	Within Frames D.F.	F	P
A	2.05	0.2201	96	0.1665	543	1.32	0.01-0.05
	4.08	0.4314	199	0.1991	1329	2.17	< 0.001
B	2.05	0.3694	93	0.1915	489	1.93	< 0.001
	4.08	0.6577	195	0.2487	1176	2.64	< 0.001

A part of the additional variance between frames in cover repetition is associated with variation in percentage cover. In general the cover repetition at a given point is greater in frames where the species occurs at most points than where it occurs at few. This is illustrated for *Carex hebes* in Table 14. In each plot, the regression of cover repetition on percentage cover is highly significant.

TABLE 13

ANALYSIS OF VARIANCE OF NUMBERS OF CONTACTS PER POINT WITH *POA CAESPITOSA* FOR POINTS WHERE THIS NUMBER DID NOT EXCEED 10

	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Between frames	116	835.7	7.20	1.60	< 0.001
Within frames	383	1719.9	4.49		

This effect is not peculiar to the species or the area. It has also been demonstrated for *Poa caespitosa* on the other plot discussed. In Table 15, the number of points with more than 10 contacts and of those with fewer are compared for frames differing in percentage cover; a significant regression is shown. This relation is doubtless due to the extensive overlapping of the foliage of different individuals that occurs when the percentage cover is high.

When variation between frames in cover repetition associated with variations in percentage cover has been eliminated, the residual variance between frames for *Carex hebes* remains highly significant, indicating that there are additional sources of local variation independent of this factor. This is also clear on a larger scale from comparison of the data from the two plots in Table 14. For a given value of percentage cover, the mean cover repetition by *Carex hebes* is almost always greater in Plot *B*.

TABLE 14

COVER REPETITION BY *CAREX HEBES* IN RELATION TO PERCENTAGE COVER (4.08 MM. PINS)

	Plot *A*		Plot *B*	
Percentage Cover	Mean Cover Repetition	No. of Frames	Mean Cover Repetition	No. of Frames
100	3.21	43	4.10	27
90	2.91	38	3.51	36
80	3.06	32	3.14	35
70	2.70	35	3.07	24
60	2.43	24	2.98	22
50	2.90	16	2.86	16
40	3.00	3	2.56	21
30	2.11	3	2.43	7
20	1.67	3	1.80	5
10	3.33	3	1.33	3
Regression mean square	7.43		55.72	
Error mean square	0.83		1.06	
F	8.97		52.55	
P	0.001-0.01		< 0.001	
b	0.0094		0.0231	

The variation between frames in cover repetition which is demonstrated above is paralleled on a larger scale by variation between adjacent strips in a plot selected for homogeneity, already mentioned in relation to Table 6. In Table 16 it is shown that for the most abundant species, *Poa caespitosa*, the proportion of points with more than 10 contacts among all those where this species was recorded varied markedly from strip to strip.

No other species is very general on this plot, but in two the variance of cover repetition has been tested. An analysis of variance was performed after square-root transformation, and the variance between the means for the five strips was compared with the variance between frames within each strip (Table 17).

The conclusion to be drawn from the results presented above, for the more abundant species anyway and probably for all were the data sufficient to prove it, is that local differences in cover repetition occur even within vegetation selected for homogeneity, and that hence the same sampling practices as those recommended in respect of percentage cover will also be appropriate for estimating cover repetition. Points arranged in frames are likely to give

less efficient estimates for the more abundant species than points distributed individually. This question has been tested directly for Plot *B* on the Bogong

TABLE 15

COVER REPETITION BY *POA CAESPITOSA* IN RELATION TO PERCENTAGE COVER

Percentage Cover	Number of Points With: 10 or Fewer Contacts	Number of Points With: More than 10 Contacts
100	30	20
90	22	14
80	59	21
70	92	27
60	112	26
50	108	22
40	41	11
30	20	7
20	12	0
10	4	1
Mean square for regression of percentage of points with more than 10 contacts on percentage cover		2921
Error mean square		473
F		6.17
P		0.01-0.05
b		2.362

High Plains, already mentioned, results with a series of single points having been compared with those for points in frames. For a given number of points, the error variance was found to differ significantly only for the dominant, *Carex hebes,* for which 423 individual points would give as accurate an estimate of mean cover repetition as 2000 points in frames. This comparison is based on an equal distribution of pins making contact among the frames in

TABLE 16

COVER REPETITION BY *POA CAESPITOSA* ON ADJACENT STRIPS, BOGONG HIGH PLAINS

Strip	Observed: 10 or Fewer Contacts	Observed: More than 10 Contacts	Expected: 10 or Fewer Contacts	Expected: More than 10 Contacts
1	76	54	66	64
2	81	81	82	80
3	117	111	116	112
4	90	67	80	77
5	34	74	55	53
	$\chi^2 = 21.58$	$n = 4$	$P < 0.001$	

which the species occurs. The fact that the variability of percentage cover causes variations in the number of points per frame for which cover repetition

data are available introduces a further complication into the estimation of mean cover repetition by the use of frames, and will tend to reduce their efficiency compared with that of individual points still further.

Table 17

VARIATION IN COVER REPETITION FOR TWO SPECIES ON ADJACENT STRIPS OF GROUND

Strip	*Hovea longifolia* Cover Repetition	*Hovea longifolia* No. of Frames	*Phebalium podocarpoides* Cover Repetition	*Phebalium podocarpoides* No. of Frames
1	3.97	11	3.73	8
2	3.90	13	4.25	9
3	2.63	8	5.19	11
4	3.00	9	4.50	13
5	4.24	9	6.96	8
Variance ratio, between strips/within strips		1.34		3.09
P		> 0.05		0.01-0.05

Thus for mean cover repetition, as for percentage cover, one may conclude that individual points are preferable to points arranged in frames, and that these points should be distributed not at random but in such a way as to ensure equal representation of all parts of the area covered. Whether or not

Table 18

CHANGES IN COVER REPETITION AS ESTIMATED BY RANDOM POINT QUADRATS AND PERMANENT POINT QUADRATS (SQUARE ROOT TRANSFORMATION)

Species	Permanent Point Quadrats: Mean, 1949	Mean, 1950	Variance of Differences	D.F. of Variance	Random Point Quadrats: Mean, 1949	Mean, 1950	Variance of Differences	D.F. of Variance
*Poa caespitosa** (var. "fine grass")	1.791	1.764	0.563	115	1.868	1.787	0.812	554
*Poa caespitosa** (var. "horny grass")	1.721	1.753	0.270	44	1.596	1.737	0.814	141
Leptorhynchus squamatus	1.545	1.507	0.516	153	1.639	1.471	0.615	433
Carex breviculmis	1.358	1.240	0.215	85	1.345	1.304	0.341	511
Celmisia longifolia	1.393	1.471	0.532	62	1.476	1.504	0.579	175
Scleranthus biflorus	2.040	1.993	1.772	22	2.050	1.936	0.838	55
Ranunculus lappaceus	1.100	1.172	0.112	20	1.183	1.134	0.147	76
Danthonia semiannularis	1.432	1.650	0.656	28	1.536	1.539	0.873	121

* Points with not more than 10 contacts in either year.

equal weight should be given to all parts of the area is a question for *ad hoc* decision; for most purposes it will probably be preferable to give weights proportional to the percentage cover by the species in question—which simply means that all points *at which the species is recorded* are given equal weight.

Again, as with percentage cover, it is to be expected that *changes* in cover repetition can better be estimated by repeated observations at fixed points than by repeated sets of random observations. This has been tested on the data from Pretty Valley (Table 18).

To facilitate comparison between the two methods, the variances in Table 18 are in each case expressed in terms of observations at a single point on each occasion. For *Poa caespitosa* the mean number of contacts at points where they were counted must again be treated separately from the proportion of points where the number of contacts exceeded 10 and was not counted. The latter values are given in Table 19. The variances in Table 19 are expressed in terms of a single frame.

TABLE 19

CHANGES IN PROPORTION OF POINTS WHERE *POA CAESPITOSA* WAS RECORDED AT WHICH THE NUMBER OF CONTACTS EXCEEDED 10, AS ESTIMATED BY TWO METHODS (ANGULAR TRANSFORMATION)

Variety	Method	Mean, 1949	Mean, 1950	Variance of Differences	D.F.
"Fine grass"	Permanent point quadrats	23.4	15.4	2841	116
	Random point quadrats	26.8	16.1	3171	278
"Horny grass"	Permanent point quadrats	12.0	7.5	1484	90
	Random point quadrats	18.8	10.8	2226	144

From Tables 18 and 19, it will be seen that the effect of permanent positions of observation, as compared with independent randomizations, on the precision of estimates of changes in cover repetition is considerably smaller than that for percentage cover (Table 7). The variances for "fine grass" in Table 19 do not differ significantly though those for "horny grass" do; in Table 18 the variances for seven of the eight species or varieties were less with permanent positions for observation—significantly so in three of the most abundant (both varieties of *Poa caespitosa*, and *Carex breviculmis*) — but in *Scleranthus biflorus* the difference was in the opposite direction, and also reached significance.

(*c*) *Distribution of Data for Cover Repetition and Their Statistical Treatment*

If the number of contacts per pin followed consistently some mathematical law of distribution, the statistical treatment of such data could be put on a much sounder basis. Moreover, if this distribution were known, a complete count of the number of contacts might be unnecessary for some purposes, since the contribution of the larger classes to the total might be deduced from the frequency of the smaller classes. Some examples of the distributions found in practice are shown in Figure 2; all classes are expressed as percentages of all points where contact with the species was made, the large zero class being omitted. This is in accordance with the general principle adopted here of treating percentage cover and cover repetition as separate variables; it is clearly possible to conceive that two plots, one of which contained one plant

only of the species under study, the other a large number of plants scattered so that their foliage did not mingle, would have the same distribution of number of contacts per pin, except for the zero class, which would differ enormously.

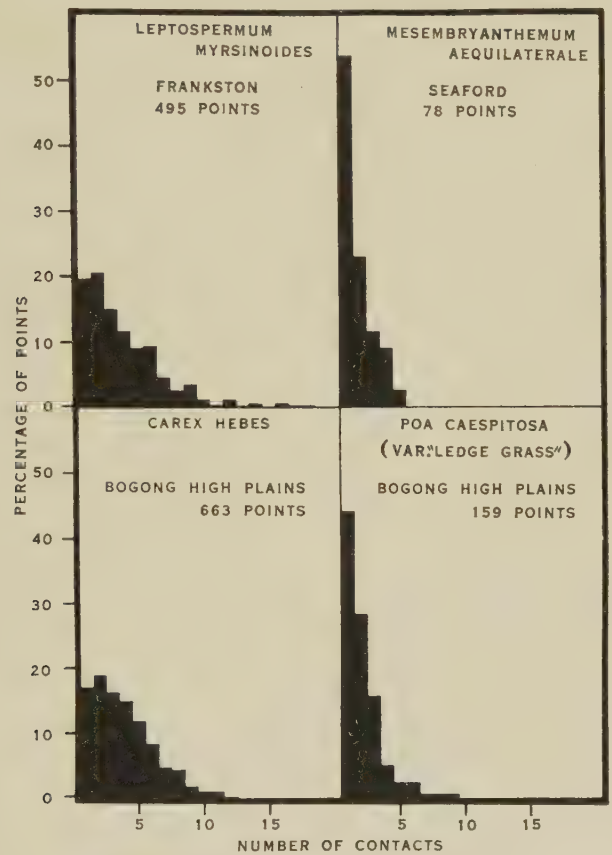

Fig. 2.—Distribution of cover repetition data for four species.

Attempts have been made to fit various types of distribution to the numerous sets of data available. The Poisson series was first tested, and was found to fit only a few cases where the cover repetition was very small; this is in accordance with expectation, for on theoretical grounds the number of contacts should follow the Poisson series only when individuals are distributed at random and the chance of more than one contact with the same individual may be neglected. Contagious distributions seemed much more promising—the distribution of projections on a horizontal plane of leaves borne on shoots spreading from a centre is clearly analogous with the situation envisaged by Neyman (1939) of larvae dispersing over a limited distance from a clump of eggs. Neyman pointed out that this implied that there was a limited area within which larvae found in any given sample area could have originated;

TABLE 20

CONTAGIOUS DISTRIBUTIONS OF NEYMAN'S TYPE *A* FITTED TO DATA FOR COVER REPETITION

No. of Contacts	*Phebalium podocarpoides* Observed	*Phebalium podocarpoides* Expected	*Ehrharta longiflora* Observed	*Ehrharta longiflora* Expected	*Rumex acetosella* Observed	*Rumex acetosella* Expected	*Poa caespitosa* ("Ledge Grass") Observed	*Poa caespitosa* ("Ledge Grass") Expected	*Mesembryanthemum aequilaterale* Observed	*Mesembryanthemum aequilaterale* Expected	*Hypolaena fastigiata* Observed	*Hypolaena fastigiata* Expected	*Leucopogon virgatus* Observed	*Leucopogon virgatus* Expected	*Carex bebes* Observed	*Carex bebes* Expected	*Leptospermum myrsinoides* Observed	*Leptospermum myrsinoides* Expected
0	61	56.5	140	139.4	17	14.3	841	839.6	23	25.7	706	706.0	798	793.0	337	311.4	504	493.8
1	3	7.2	22	20.2	9	12.2	70	62.8	42	33.2	144	130.0	70	52.5	112	129.6	97	76.1
2	8	10.2	14	17.8	7	9.6	45	49.4	18	23.0	74	87.5	41	57.9	125	143.5	102	102.2
3	8	10.0	10	11.2	9	6.3	25	27.1	9	11.2	39	43.9	33	43.8	107	122.2	74	96.4
4	11	8.0	8		4		8	12.0	7		21	19.3	29	26.2	99	92.8	57	74.5
5	6	5.7	4		2		4		2		8	8.0	11	13.6	78	67.1	45	52.4
6	5	5.4	1		2		4		0		3		7	6.7	55	46.9	46	35.6
7	3		1	11.4	0	7.6	1		0		3		3		31	31.8	23	24.0
8	0		0		0		1	9.1	0	7.9	1	5.3	4		29	20.8	13	16.0
9	1	6.0	0		0		1		0		1		1	6.3	12	13.3	17	10.5
10	2		0		0		0		0		0		1		7	20.6	6	6.7
>10	1		0		0		0		0		0		2		8		15	10.8
χ_2	6.75		1.79		3.20		3.11		4.29		5.67		18.10		16.32		25.67	
P	> 0.05		> 0.05		> 0.05		> 0.05		> 0.05		> 0.05		< 0.01		< 0.05		< 0.01	

if the distribution within this larger area of the probability of origin of larvae found within the sample area were known, the distribution of total numbers of larvae in the sample area could be derived. Making three different assumptions regarding the distribution of the probability of origin of the larvae, he derived three types of distribution for numbers in the sample area. In spite of the artificiality of the underlying assumption, his Type *A* distribution has in practice been found to fit adequately many groups of observational data, not only from entomology (Beall 1940), but also for the numbers of individual plants within small areas of natural vegetation (Archibald 1948). It was hoped that it would also fit the distribution of numbers of contacts in point quadrat data.

In Table 20 are shown some examples of contagious distributions of Neyman's Type *A* fitted to data for cover repetition. Where a satisfactory fit was not obtained by Neyman's method of moments, Shenton's (1949) maximum likelihood method was applied. It will be seen that, though several sets of data could be adequately fitted by this distribution, three could not.

A number of other distributions were tested, among them Neyman's Types *B* and *C*, the double Poisson series of Thomas (1949), the negative binomial series, and the truncated log-normal distributions of Thompson (1950). None gave consistently more successful results than Neyman's Type *A*, and most were considerably worse.

It seemed likely that the difficulties in fitting might well arise from the fact already mentioned that, from the biological point of view, the distribution of numbers of contacts where the species is present at all should be independent of the size of the zero class. Accordingly, attempts were made to fit distributions to the data, treating the zero class as unknown; in other words, one could assume that the zero class of the observational data was made up of one part properly belonging to the distribution under test—perhaps that part falling within the outlines formed by joining the extreme branches of each individual—and another part unrelated to that distribution and depending on percentage cover. The number of points actually in the zero class was accordingly ignored, and arbitrary smaller numbers inserted, that giving the best fit with the distribution under study being found by trial and error. With the negative binomial distribution, a good fit was invariably obtained by this procedure. Some examples are shown in Table 21.

It has already been pointed out that there are species of plants, notably the tussock grasses, in which a complete count of contacts with a vertical pin is impracticable where the contacts are numerous. Even in species where a complete count is practicable, it may be undesirably laborious, and a method of estimating the mean cover repetition from the frequency of points with a few contacts only would be welcome for routine use where speed is more important than precision. Since it has been shown that, apart from the "contaminated" zero class, cover repetition data can be satisfactorily fitted by a negative binomial distribution, if such a distribution could be fitted taking into account only the first few classes an estimate could be obtained of the

TABLE 21

NEGATIVE BINOMIAL DISTRIBUTIONS FITTED TO DATA FOR COVER REPETITION, IGNORING THE ZERO CLASS

No. of Contacts	*Phebalium podocarpoides* Observed	*Phebalium podocarpoides* Expected	*Monotoca scoparia* Observed	*Monotoca scoparia* Expected	*Poa caespitosa* ("Ledge Grass") Observed	*Poa caespitosa* ("Ledge Grass") Expected	*Hypolaena fastigiata* Observed	*Hypolaena fastigiata* Expected	*Leucopogon virgatus* Observed	*Leucopogon virgatus* Expected	*Carex hebes* Observed	*Carex hebes* Expected	*Leptospermum myrsinoides* Observed	*Leptospermum myrsinoides* Expected
0		(1.5)		(11.0)		(105.2)		(230.1)		(80.3)		(48.0)		(79.8)
1	3	11.5	19	16.5	70	72.4	144	142.8	70	67.6	112	100.9	97	100.2
2	8		16	16.5	45	41.4	74	76.3	41	48.0	125	126.4	102	94.3
3	8	8.5	9	13.8	25	22.2	39	38.6	33	31.9	107	122.7	74	78.9
4	11	8.1	12	10.3	8	11.4	21	18.9	29	20.6	99	101.8	57	61.9
5	6	6.7	7	7.2	4	5.8	8	9.1	11	13.0	78	75.9	45	46.6
6	5	5.0	5	4.8	4		3		7	8.1	55	52.3	46	34.1
7	3		4		1		3		3	5.0	31	33.9	23	24.4
8	0		1		1	5.6	1	8.2	4		29	20.9	13	17.2
9	1	8.2	3	7.9	1		1		1	7.5	12	12.4	17	12.0
10	2		1		0		0		1		7	7.2	6	8.3
> 10	1		0		0		0		2		8	8.6	15	17.3
χ^2	1.34		2.52		2.66		0.44		4.87		6.94		9.77	
P	> 0.05		> 0.05		> 0.05		> 0.05		> 0.05		> 0.05		> 0.05	

frequencies of the higher classes, and hence of the mean cover repetition. Unfortunately, a method of fitting a negative binomial distribution to data comprising a limited range of classes only has yet to be devised, and in the absence of such a method it is necessary to proceed empirically. It has been found that the frequencies in the lower classes bear a fairly constant relationship to the mean—not only in different sets of data for a single species, but also in different species.

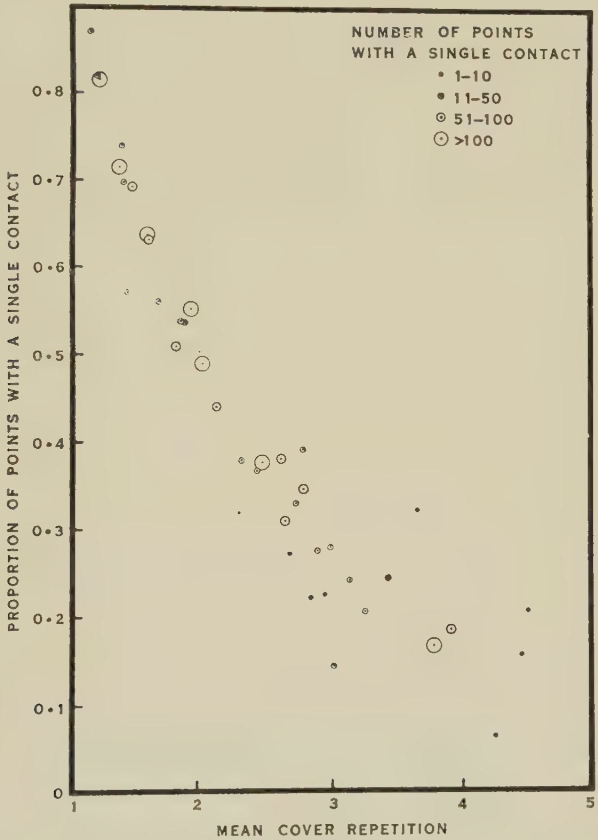

Fig. 3.—Relation of mean cover repetition to proportion of pins with a single contact only.

In Figure 3, the mean cover repetition has been plotted against the proportion of points with only a single contact. The data for this diagram have been taken from 5500 records on 31 species in four types of vegetation—heath, mountain grassland, sand dunes, and waste land. It will be seen that the relationship is close—at least where the number of points with a single contact is large enough to give a reasonably satisfactory estimate of the proportion of such points. A curve such as that of Figure 3 could clearly be used to estimate the mean cover repetition if only points with a single contact had been counted; for higher values of cover repetition, however, the precision of

such an estimate would be low. The precision could be improved by taking, instead of the unit class only, the proportion falling into a number of the lower classes together. This has been done in Figure 4 for the points with from one to four contacts inclusive; it will be seen that the extreme error in the estimation of mean cover repetition from such a curve would be unlikely to exceed 10 per cent. Curves of this type would, then, provide a way out of the impasse presented by plants like *Poa caespitosa* where counting of the larger numbers of contacts is impracticable; it should be borne in mind, however, that the tussock habit of these plants, which prevents a direct measure of cover repetition, may also result in these species not obeying the same rule as those included in Figures 3 and 4. However, *faute de mieux,* it may sometimes be necessary to obtain estimates in this way of mean cover repetition, and of the mean number of contacts at points where they cannot well be counted. The procedure may be illustrated by a series of observations on the "ledge grass" variety of *Poa caespitosa,* for which the distribution was:

Contacts	Points
1	68
2	38
3	28
4	9
5	6
6	5
7	3
Numerous, uncounted	7

The proportion of points with from one to four contacts was 0.872; from the curve in Figure 4 this would correspond with a mean cover repetition of 2.63—which in turn would indicate an average number of contacts of 12.3 for those points at which they had not been counted.

The facts that the observational data consist of integers, generally small, that their distribution is very skew, and that the variance is not independent of the mean suggest that the usual statistical techniques for normally distributed data would not be appropriate without modification. A transformation is required that can be applied to the data to render their distribution more nearly normal and equalize their variance, and hence to make it possible to use the usual tests of significance. Anscombe (1948) has proposed a hyperbolic sine transformation for the negative binomial distribution, but it seems doubtful whether that would be applicable where the zero class is ignored, as in the present case. For many types of skew data, it has empirically been found that square root transformations are satisfactory; they were likewise found to give reasonably good results with cover repetition data. As an example of the effect of this procedure, the results for *Carex hebes* on Plot *B* (Table 14) may be cited. It has already been shown that the mean cover repetition for this plant differs for frames with different percentage cover. Accordingly, the variance between frame means has been computed separately for the frames with seven or more records for this species, and for those with six or fewer. The results are shown in Table 22. It will be seen that whereas

the difference in means was in the untransformed data accompanied by a difference in variance, after transformation the variances were almost identical.

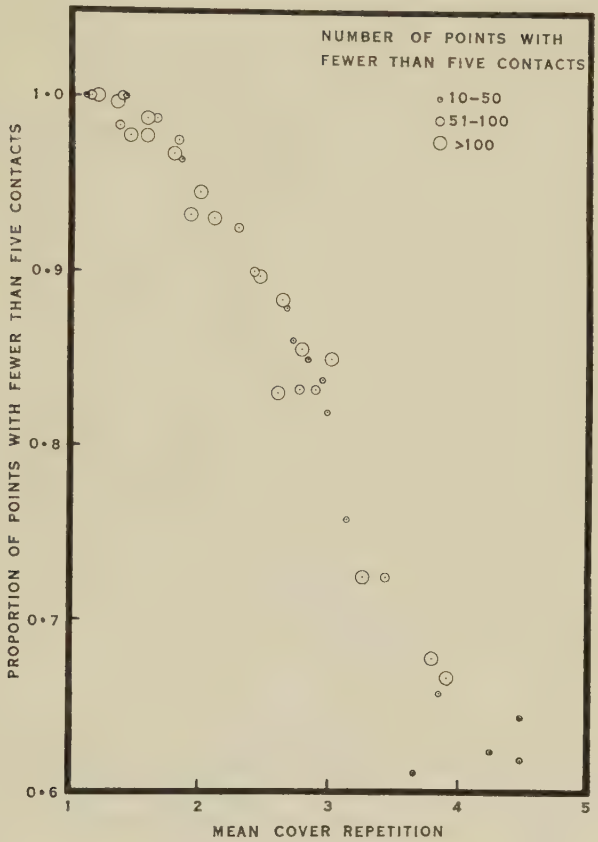

Fig. 4.—Relation of mean cover repetition to proportion of pins with fewer than five contacts.

One further question in connection with the statistical treatment of data for cover repetition needs to be mentioned: when a mean is being computed for a plot of ground, should values from different parts of this plot be weighted in proportion to their area, or in proportion to the product of area and percentage cover? The latter seems the more logical procedure for most purposes, allowing each part of the plot where the species occurs to contribute equally to the mean, whereas the former procedure would give much greater weight to isolated individuals than to others forming a continuous cover. Accordingly, in this paper values from equal areas have been weighted in proportion to the percentage cover. For certain purposes, though—as, for instance, where one has reason to suspect a change in plant habit along a local gradient of climatic or edaphic factors—equal weighting of equal areas may be more appropriate.

(*d*) *The Personal Factor*

Personal differences between observers might be expected to be more important in determining cover repetition than percentage cover—the scope for variation in counting contacts is greater than in determining whether or not contact has occurred.

Table 22

EFFECT OF SQUARE ROOT TRANSFORMATION ON VARIANCE OF COVER REPETITION FOR *CAREX HEBES*

	Variance Among Frames With			
	1-6 Records	7-10 Records	*F*	*P*
Untransformed data	4.959	10.551	2.128	< 0.001
Transformed data	0.590	0.615	1.043	> 0.05

The data collected for Table 9 were examined in respect of cover repetition, only those points being taken into account at which the species was recorded by all three observers. The data were subjected to analysis of variance, after square root transformation (Table 23). Only in one species were significant differences between observers demonstrated. In view, however, of the much smaller number of observations than for percentage cover, it is hardly possible to draw conclusions as to the relative importance of personal variation in estimating these two characteristics.

Table 23

THE PERSONAL FACTOR IN ESTIMATING COVER REPETITION

Species	No. of Points	Mean Cover Repetition as Estimated by Observer *A*	*B*	*C*	Significance (*P*) of Differences Among Observers
Lolium perenne	138	3.17	3.70	3.41	0.001-0.01
Plantago lanceolata	136	2.24	2.40	2.39	> 0.05
Bromus spp.	48	2.15	2.04	2.15	> 0.05
Cryptostemma calendulaceum	57	1.88	2.18	2.14	> 0.05
Romulea bulbocodium	9	2.78	2.78	2.89	> 0.05
Medicago arabica	13	1.62	1.54	2.00	> 0.05
Medicago denticulata	7	2.00	2.29	2.43	> 0.05

IV. Estimation of Percentage of Sward

The third type of measurement for which point quadrats may be used is a determination of the proportion each species forms of the total quantity of vegetation. This is often termed "percentage of sward," and is generally assessed in terms of dry weight. It has been claimed that point quadrats can provide a good estimate of this—that the number of contacts of pins with

one species as a proportion of the total number of contacts with all is closely related to the proportion of dry matter provided by this species (see Levy 1933; Levy and Madden 1933; Charpentier and Saarela 1941; Arny and Schmid 1942; Arny 1944; Eden and Bond 1945). With this question we shall not concern ourselves here, though it is plain that where herbaceous and woody plants grow together such a relationship cannot be expected to hold between them, and there is considerable evidence that even in a purely herbaceous community the relation is far from constant. Be this as it may, it would seem that this ratio of contacts with one species to contacts with all has some claim to be regarded, in its own right, as a measure of the part played by a species in the vegetation, irrespective of its relation to dry weight or any other measure; accordingly we shall proceed to discuss the derivation and treatment of data for "percentage of sward," defined as

$$\frac{(\text{Percentage cover}) \times (\text{cover repetition}) \text{ for one species}}{\text{The sum for all species of } (\text{percentage cover}) \times (\text{cover repetition})} \times 100.$$

As estimated by the point quadrat method, this is of course equivalent to

$$\frac{\text{Number of contacts with one species}}{\text{Number of contacts with all species}} \times 100.$$

TABLE 24

PERCENTAGE OF SWARD FORMED BY DIFFERENT SPECIES, AS ESTIMATED BY PINS OF DIFFERENT DIAMETERS

Plot	Species	Mean Percentage of Sward		t	P
		2.05 mm. Pin	4.08 mm. Pin		
A	*Carex hebes*	71.00	69.80	0.48	> 0.05
	Poa caespitosa	4.77	6.68	1.52	> 0.05
	Rumex acetosella	4.16	5.26	1.15	> 0.05
	Viola betonicifolia	5.47	6.13	0.84	> 0.05
B	*Carex hebes*	54.59	62.91	2.31	0.01-0.05
	Poa caespitosa	11.28	7.49	1.99	0.01-0.05
	Asperula gunnii	6.61	5.23	0.78	> 0.05
	Viola betonicifolia	2.20	2.72	0.78	> 0.05
	Microseris scapigera	3.90	3.31	0.51	> 0.05

(a) *Size of Pin*

It is to be expected that the estimates of percentage of sward will be much less affected by pin size than either percentage cover or cover repetition. Since the variation in estimates of cover repetition with varying pin size tends to be greater for microphyllous than macrophyllous species, one would expect percentage of sward for the former to be slightly over-estimated, that for the latter underestimated, from the proportion of contacts with a coarse pin. That such effects may, however, usually be ignored is shown by Table 24, which presents the estimates of percentage of sward calculated from the same data as Tables 2 and 11. Only in two cases do the differences just reach significance, and it seems likely that these may well be the result of chance variation.

(*b*) *Distribution of Points*

Since percentage cover and cover repetition had been shown to vary from point to point over an area, it was to be expected that the figures for percentage of sward would likewise vary. This is shown in Table 25, where the variances within and between frames are given for the data from Plot *B* discussed above (those obtained with broad pins). The level of significance reached is so high that further demonstration is hardly necessary. These results imply, of course, that arrangement of points in frames leads to inefficient use of the observers' time—even more so than in estimating percentage cover. It may be computed from these data that the number of individual points giving estimates of equal precision to the means for 200 frames each with 10 points would range from 423 for *Asperula gunnii* to 704 for *Poa caespitosa*.

TABLE 25

ANALYSIS OF VARIANCE OF DATA FOR PERCENTAGE OF SWARD, BOGONG HIGH PLAINS

Species	Weighted Variance Between Frames (D.F. 199)	Weighted Variance Within Frames (D.F. 1603)	*F*	*P*
Carex hebes	28441	4125	6.89	< 0.001
Poa caespitosa	5134	1610	3.19	< 0.001
Asperula gunnii	6133	854	7.18	< 0.001
Viola betonicifolia	1196	371	3.22	< 0.001
Microseris scapigera	2444	562	4.35	< 0.001

In addition to the variation in percentage of sward between frames, variation is also, as one might expect, found to occur between larger areas within apparently homogeneous vegetation. In the plot divided into strips already discussed in connection with Tables 6, 16, and 17, an analysis of variance of the data for percentage of sward gave the results in Table 26. In deriving these percentages, at points where the contacts with the dominant "fine grass" variety of *Poa caespitosa* were too numerous to be counted they were taken as 10, a figure derived for this variety by the procedure described on pp. 29-30. It will be seen that two of the four species show significantly greater variance between strips than within strips between frames.

Thus we come to the conclusion that for percentage of sward, as for percentage of cover and cover repetition, individual points are to be preferred to points arranged in frames, and they should be distributed over the area under study in such a way as to ensure equal representation of all parts of it; furthermore, records should be kept in a manner that will enable the data to be reassembled in different ways, so that the variation over the area may be studied.

The results already described for percentage cover and for cover repetition lead one to suppose that *changes* in percentage of sward, too, can be more accurately estimated from permanent point quadrats than from points distributed independently for each occasion of observation. This has been

tested on the data from Pretty Valley, Bogong High Plains, and the results are shown in Table 27. Records for *Poa caespitosa* of more than 10 contacts at a point have been treated in the way described on pp. 29-30. In all species but one the change is estimated more precisely by permanent point quadrats, and in the more abundant species the difference is very marked. It may, then, be concluded that, for determination of changes in the composition of the "sward," observational labour will be more economically used if the same points are used in successive years than if the points are distributed afresh at random in each year.

TABLE 26

VARIATION IN PERCENTAGE OF SWARD FORMED BY FOUR SPECIES ON ADJACENT STRIPS OF GROUND

Species	Strip 1	2	3	4	5	Weighted Variance Between Strips (D.F. 5)	Weighted Variance Within Strips (D.F. 95)	*F*	*P*
Hovea longifolia	12.2	8.3	2.0	4.0	10.6	2.51	0.88	2.85	0.01-0.05
Poa caespitosa ("ledge grass")	0.8	1.1	0.1	0.4	1.2	0.0308	0.0314	1.02	>0.20
Phebalium podocarpoides	9.5	6.0	7.4	10.0	14.2	1.13	1.72	1.52	>0.20
Carex breviculmis	1.1	0	0	0.6	0.3	0.0245	0.0095	2.58	0.01-0.05

(*c*) *Statistical Treatment of Results*

Where for any purpose—for instance, forage studies—an estimate of the percentage composition of the total amount of vegetation over an area is required, it will clearly be necessary to use a weighted mean in combining data for percentage of sward from different parts of the area, the weights being proportional to the total quantity of vegetation. In practice, this means that the weights are proportional to the total number of contacts with all species within each portion of the area. This procedure has been used for all "percentage of sward" data in this paper, and variance estimates are weighted correspondingly. For certain purposes, however, it may be preferable to use weights proportional to areas rather than to total numbers of contacts.

As with results for percentage cover, those for percentage of sward will not have variance independent of their mean, but it may be expected that their sampling variance will be equalized by angular transformation. In Table

28 are shown the results of applying this transformation to the data already used in Table 26. The differences among the variances in different strips have been reduced by the transformation, but remain significant as judged by the

TABLE 27

CHANGES IN PERCENTAGE OF SWARD AS ESTIMATED BY RANDOM AND PERMANENT POINT QUADRATS

Species	Permanent Point Quadrats			Random Point Quadrats		
	Mean, 1949	Mean, 1950	Variance*	Mean, 1949	Mean, 1950	Variance*
Poa caespitosa (var. "fine grass")	55.70	51.68	4.7183	51.24	54.80	13.5607
Poa caespitosa (var. "horny grass")	9.62	13.64	2.2559	14.34	10.93	8.3416
Leptorhynchus squamatus	10.09	10.04	1.0303	10.79	9.77	3.8896
Carex breviculmis	7.33	6.99	0.6672	7.77	6.34	0.9981
Celmisia longifolia	2.75	3.78	0.3915	2.07	3.97	0.8420
Scleranthus biflorus	1.83	1.63	0.3547	1.02	2.20	0.6137
Ranunculus lappaceus	0.72	1.19	0.0923	0.62	0.89	0.0844
Danthonia semiannularis	1.80	2.15	0.5606	2.17	2.43	0.7161

* To facilitate comparison, the variance in each case is that of the difference between the means in 1949 and 1950 of samples of 100 frames (1000 points). The variance estimates for permanent point quadrats are based on 109 degrees of freedom, those for random point quadrats on 296 degrees of freedom.

Bartlett test. It has already been shown that variance between frames exceeds the sampling variance (that within frames), and it is clear that this additional component of the variance is not equalized by the transformation.

TABLE 28

WEIGHTED VARIANCE OF ESTIMATES OF PERCENTAGE OF SWARD, BEFORE AND AFTER ANGULAR TRANSFORMATION

Strip	*Hovea longifolia*		*Phebalium podocarpoides*	
	Untransformed	Transformed	Untransformed	Transformed
1	12960	13476	16487	15589
2	9770	11227	7455	7928
3	2020	4551	14938	14596
4	3240	5348	13789	12282
5	16050	17876	53892	35814
χ^2	26.3	12.6	21.8	10.3
P	< 0.001	0.01-0.05	< 0.001	0.01-0.05

(*d*) *The Personal Factor*

Idiosyncrasies of observers affect the results obtained for percentage of sward, as well as those for percentage cover and cover repetition. In Table 29 are presented the results for percentage of sward from the collection of

data already used for Tables 9 and 23. Records at 288 points (those at which all observers recorded contact with at least one species) were available for this purpose, and the results of the weighted analysis of variance are indicated in the last column of the table.

TABLE 29

PERSONAL FACTOR IN ESTIMATING PERCENTAGE OF SWARD

Species	Percentage of Sward as Estimated by Observer			Significance (*P*) of Differences Among Observers
	A	*B*	*C*	
Lolium perenne	41.1	45.6	43.7	0.001-0.01
Plantago lanceolata	26.0	25.2	25.6	> 0.05
Bromus spp.	13.5	9.2	10.6	< 0.001
Cryptostemma calendulaceum	8.7	10.2	10.0	0.01-0.05
Romulea bulbocodium	3.4	2.6	2.4	0.01-0.05
Medicago arabica	2.1	1.8	2.7	0.01-0.05
Medicago denticulata	2.4	1.7	2.4	> 0.05

In five of the seven species studied, the observers differed significantly in their estimates of percentage of sward. Except for *Medicago arabica*, however, the main contribution to this effect was from Observer *A*, and the differences between Observers *B* and *C* were much smaller—a result similar to that already mentioned in respect of percentage cover.

Crocker and Tiver (1948) failed to find significant differences between their three observers, using a method of calculation essentially similar to that for percentage of sward. It must be noted that the method used here provided a highly sensitive measure of personal differences, in view of the fact that each observer recorded at precisely the same points; though it is not explicitly stated, it would appear that in Crocker and Tiver's work different positions for the frames were used for each observer, which greatly reduced the sensitivity of their test.

It is clear, then, that the point-quadrat method is not quite as objective as has been claimed, and that personal differences cannot be ignored, though the differences demonstrated between observers are small compared with those commonly studied in vegetation.

V. DISCUSSION

The first conclusion that may be drawn from the results presented is that the pins used in point quadrat work should be as fine as is practicable, and that where data for percentage cover only are required an optical apparatus is preferable to a rigid pin, which must lead to over-estimation of percentage cover and cover repetition; figures for percentage of sward are much less sensitive to pin size. Where the principal interest centres in *changes* in the vegetation, the use of a thicker pin is probably less objectionable — changes recorded will be in the correct direction, though their magnitude will often not provide a measure of the true extent of the changes occurring. An optical method would, of course, be essential for use with the tree layer.

The use of oblique rather than vertical pins (see Introduction) would appear to be unobjectionable provided information only on percentage of sward is required, and an appropriate definition of this quantity is accepted. Since most plants include vertical and oblique, as well as horizontal, organs, the use of oblique pins certainly increases the number of contacts per point and hence the precision obtained with a given number of points, but information on percentage cover and cover repetition is lost. Incidentally, since the vertical component in the distribution of organs differs from species to species, it is to be expected that the figures for percentage of sward obtained by the two methods would not be identical; since, however, the definition of percentage of sward adopted for its estimation by vertical pins is arbitrary, and the quantity is intended merely as an index of the proportional composition of the vegetation, figures obtained with inclined pins have no less validity than those with vertical pins.

It is clear that a great deal of time and effort has been wasted in the past through the use of frames with 10 places for pins instead of individual placement of each pin. Blackman suggested the latter technique in 1935, but the only published work in which it has been used seems to be that of Eden and Bond (1945) and Bond (1947) in Ceylon. However, in assessing the advantages of individual points as against groups, it must not be forgotten that the time occupied in placing each pin at random is greater in the former, and the most economical procedure could be worked out precisely only if information were available both of the rates of work by the two methods and of their relative precision in the particular problem under investigation. Moreover, the scope for subconscious choice of placement is greater with individual points than with frames, and special care must be taken to avoid it. This difficulty could be eliminated by using frames in each of which only one point is recorded, the position of that point in the frame being determined at random.

Fresh random distributions of points on each occasion, when changes in the vegetation are the main subject of interest, are an extremely uneconomical procedure, and should be abandoned in favour of fixed positions for the points to be observed on each pair of successive occasions. The points should be marked, not by a pin or peg permanently placed there, which might affect the growth of the plants, but by measurement from pegs at some distance. With the exception of Arnborg (1943, 1949), previous investigators appear not to have recognized the advantages of performing successive observations at the same points.

The personal differences demonstrated between observers, although not large, show that it is desirable as far as possible to use the same observers throughout a series in which comparisons are to be made. If this is impracticable, supplementary comparisons of results obtained by the different observers on the same vegetation should be included, in order that the magnitude of the differences among them may at least be known, and perhaps corrected.

Several investigators who have used the point quadrat method have attempted to determine the number of points required to give a trustworthy analysis of the vegetation. This is not a question that can be answered in general terms. The answer not only will depend on the degree of precision required, but will vary from species to species (particularly as between more abundant and less abundant species), and will not be the same for determinations of percentage cover as for percentage of sward. The question can be answered only in relation to the particular problem and the particular type of vegetation involved.

As regards percentage cover, if points are distributed at random an answer may readily be given from first principles. The standard deviation of values obtained by use of k points will be $\sqrt{\frac{pq}{k}}$ where p is the percentage cover and q its complement $(100 - p)$. If, for instance, a standard error of 10 per cent. of the mean were considered satisfactory, this would be attained for a species occupying half the area with the use of 100 randomly distributed points; but for a species occupying only one-tenth of the area, 900 points would be necessary, and for one occupying one-hundredth, 9900 points.

For percentage cover if the points are not distributed at random, and for density of cover and percentage of sward in any circumstances, an answer cannot be given *a priori* to the question of how many points are required to give a specified degree of precision, but must be derived from observations directly on the vegetation concerned. In general, if the area under study be divided into small portions and two points placed at random be recorded within each portion, the variance between the points of a pair will indicate roughly the number of points required to produce results of a given precision. For observations on changes in vegetation in the course of time, without knowing how variable the changes in fact are it is impossible to obtain a firm figure for the number of points needed to estimate them with the accuracy required, and any estimate based on observations on a single occasion is bound to be no more than guesswork.

In general, if the same points are used for recording abundant and infrequent species, the relative precision of estimates obtained for the former will necessarily be greater than for the latter. While estimates of equal relative precision for the less abundant as for the more abundant species may be desirable, they will not usually be practicable, and a compromise will be necessary.

Although a critical approach to the point-quadrat method has been adopted, and attention has been drawn to some of the difficulties in applying it, the general conclusions that emerge are not adverse to the method. On the contrary, it remains one of the most trustworthy methods available to the ecologist, and one of the most nearly objective. With the improvements suggested here, one need be in no doubt that the method will in future serve ecology even better than it has done in the past.

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ENZYMES OF *ASPERGILLUS ORYZAE*

I. THE DEVELOPMENT OF A CULTURE MEDIUM YIELDING HIGH PROTEASE ACTIVITY

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Summary

When grown on modified Raulin's medium, containing additional sucrose, ammonium tartrate, and phosphate, *Aspergillus oryzae* yields more protease than when cultivated on unmodified Raulin's or Czapek-Dox media. Replacement of sucrose in the medium by fructose, invert sugar, glucose, maltose, starch, or lactose decreases the yield of enzyme in that order. Ammonium tartrate may be replaced by equivalent concentrations of the more readily available salts, sodium potassium tartrate (Rochelle salt) and ammonium chloride, without loss in activity.

Varying the concentration of each of the constituents has shown that the following medium is the one most favourable for protease formation: 4.0 per cent. sucrose, 3.0 per cent. sodium potassium tartrate, 1.1 per cent. NH_4Cl, 0.3 per cent. K_2HPO_4, 0.05 per cent. $MgSO_4.7H_2O$, 0.002 per cent. $FeSO_4.7H_2O$, and 0.1 p.p.m. Zn. The pH is adjusted to 6.2 with HCl. With this medium, maximum yield of enzyme is obtained in 10 days at 22°C. with a ratio of volume to surface area of 0.9.

I. INTRODUCTION

It has been shown that a strain of the mould *Aspergillus flavus-oryzae*† produces high yields of proteolytic enzymes when grown on steamed wheat bran (Maxwell 1950). To purify these enzymes it was considered advisable to develop a protein-free liquid culture medium on which the mould would grow vigorously and produce high concentrations of extra-cellular enzymes.

Twenty-five strains of *A. oryzae* were tested for growth and proteolytic activity on various liquid media. The Czapek-Dox and Raulin media were found to support growth of the organism with the production of small amounts of protease. Strain 292.4795, from the collection of Dr. C. Thom, which had previously been found to produce the highest yields of protease when grown on steamed wheat bran, also proved most suitable for surface culture on liquid media.

This paper describes changes in the production of protease by this mould with variations in the nature and concentration of components of the medium. From these data a medium has been selected for further studies on the enzymes of *A. oryzae*.

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† Referred to in this series of papers as *A. oryzae*.

II. Methods

Cultures of *A. oryzae* were prepared by inoculating sterile medium with 0.5 per cent. by volume of a heavy spore suspension of the organism, and incubating at the prescribed temperature. The resulting cultures were filtered, followed by estimation of protease activity of the medium, using the gelatin viscosity reduction method of Lennox and Ellis (1945) and the gravimetric method of W. G. Crewther (in press). The latter method expressed protease activity in terms of the weight of gelatin rendered soluble in 80 per cent. ethanol as a result of protease action. The activity is expressed as mg. of gelatin and may be converted to enzyme units by reference to a standard curve.

Sucrose was estimated by inversion with acid and application of Bertrand's method (1906).

Ammonia nitrogen was determined by direct distillation from alkaline solution and titration, and total nitrogen by digestion with sulphuric acid and Nesslerization.

III. Selection of Medium

In order to determine the composition of a basal medium which could be modified to improve the yield of protease produced by *A. oryzae,* this organism was grown on a number of modifications of Czapek-Dox and Raulin media, one of the latter being that developed by F. G. Lennox (personal communication). The protease activities developed in 14 days at 25-27°C. were compared with the activity of an aqueous extract of a steamed-bran culture of the same organism. Both viscometric and gravimetric methods were used (Table 1). The tartrate medium of Lennox is seen to be considerably better than the other modifications tested, and it has accordingly been used as the basal medium for further investigation. The concentration of each of the ingredients, shown in Table 1, has been changed and combined with that of other ingredients in different ways in order to obtain the best possible yield of protease at 25-27°C. The effects of varying the carbon source and the organic anion, and of the importance of trace metals in the medium were also investigated.

(*a*) *Variation of the Carbon Source*

Table 2 provides figures for the protease activity of cultures of *A. oryzae* grown on the basal medium in which the sucrose was substituted with other sugars or starch. Both the viscometric and gravimetric methods showed sucrose to give the highest protease activities. Experiment has shown this superiority of sucrose to be maintained when the basal medium is changed in other ways.

(*b*) *Concentration of Ammonium and Tartrate Ions*

The ammonium tartrate in the basal medium was replaced by varying amounts of ammonium chloride and tartaric acid, the pH being adjusted to

TABLE 1

PROTEASE ACTIVITIES ON LIQUID MEDIA

Medium	Composition of Medium (g./100 ml.)														Time of Incubation at 25-27°C. for Maximum Yields of Protease (days)	Maximum Protease	
	Sucrose	Molasses	K Acetate	NH_4 Tartrate	NH_4NO_3	Urea	Gelatin	K_2HPO_4	KCl	NaCl	$MgSO_4.7H_2O$	$FeSO_4.7H_2O$	$ZnSO_4$	$MnSO_4$		Viscometric Method (units/ml.)	Gravimetric Method (units/ml.)
Czapek-Dox	1.0	—	—	—	0.3	—	—	0.1	0.05	—	0.05	0.001	—	—	10	0.28	56
																0.31	68
Modified Czapek-Dox	1.0	—	—	—	—	—	1.0	0.1	0.05	—	0.05	0.001	—	—	10	0.63	108
																0.56	122
Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.61	224
																0.61	240
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.05	0.05	0.2	0.025	0.0002	—	—	8	0.56	394
																0.53	230
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.1	0.05	0.2	0.025	0.0002	—	—	8	0.50	232
																0.48	158
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	0.2	0.05	0.0002	—	—	8	0.90	274
																0.63	280
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	0.5	0.025	0.0002	—	—	8	0.48	200
																0.44	146
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	1.0	0.025	0.0002	—	—	8	0.40	218
																0.30	140

TABLE 1 (*Continued*)

PROTEASE ACTIVITIES ON LIQUID MEDIA

Medium	Composition of Medium (g / 100 ml.)														Time of Incubation at 25-27°C. for Maximum Yields of Protease (days)	Maximum Protease	
	Sucrose	Molasses	K Acetate	NH_4 Tartrate	NH_4NO_3	Urea	Gelatin	K_2HPO_4	KCl	NaCl	$MgSO_4.7H_2O$	$FeSO_4.7H_2O$	$ZnSO_4$	$MnSO_4$		Viscometric Method (units/ml.)	Gravimetric Method (units/ml.)
Modified Raulin's medium	1.0	—	—	1.0	—	—	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.31 0.29	148 124
Modified Raulin's medium	1.0	—	—	0.5	—	0.5	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.11	36 38
Modified Raulin's medium	1.0	—	—	0.5	—	—	—	0.03	0.05	0.2	0.025	0.0002	0.0005	0.0002	8	0.28	174
Modified Raulin's medium	1.0	—	0.05	0.5	—	—	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.26	152
Modified Raulin's medium	—	2.0	—	0.5	—	—	—	0.03	0.05	0.2	0.025	0.0002	—	—	8	0.36	234
Tartrate medium (Lennox)	1.0	—	—	1.0	—	—	—	0.1	0.05	—	0.05	0.002	—	—	8	2.90 2.88	262 252
Bran digest	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11	3.88 2.78 5.00	380 480 440

6.2 with potassium hydroxide. When grown on such media, *A. oryzae* was found to produce maximum protease activity when the ratio of ammonium chloride to tartaric acid was between 1.4 and 1.7 (Fig. 1). The use of these two salts in the ratio of 1.4 approximates to the addition of ammonium tartrate, as such, to the medium.

TABLE 2

EFFECTS OF CARBOHYDRATES IN THE MEDIUM

Media contain the following percentages of nutrients: carbohydrate, 1; ammonium tartrate, 1; K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; $FeSO_4.7H_2O$, 0.02. pH 6.2

Carbohydrate	Time for Maximum Protease Production (days)	Protease Activity (units/ml.) Viscometric Method	Protease Activity (units/ml.) Gravimetric Method	Growth of Mould
Starch	13	1.48	350	Slow during the first 5 days, then very heavy
Sucrose	8	2.65	370	Heavy mycelial mat developed by third day
Fructose	8	2.43	358	Heavy mycelial mat developed by third day
Invert sugar	8	2.14	358	Heavy mycelial mat developed by third day
Glucose	8	2.06	328	Heavy mycelial mat developed by third day
Maltose	8	1.75	262	Heavy mycelial mat developed by third day
Lactose	12	0.88	268	Heavy mycelial mat developed by third day

(c) *Concentration of Ammonium Tartrate and Sucrose*

The optimum ratio of sucrose to ammonium tartrate was determined by growing the mould on media containing varying concentrations of sucrose. For each sucrose concentration, media containing 0.1, 0.5, 1.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 per cent. ammonium tartrate were tested for protease production. The optimum tartrate concentration, the maximum protease activity, and the weight of mycelium corresponding with each sucrose concentration are shown in Table 3. With the exception of media containing less than 1.0 per cent. sucrose, highest yields of protease were obtained when the ratio

of sucrose to ammonium tartrate was of the order of 2 : 1 by weight. Maximum protease activity was obtained with a concentration of 4 per cent. sucrose, greater concentrations giving rise to higher mycelium weights but rather slower production of the protease.

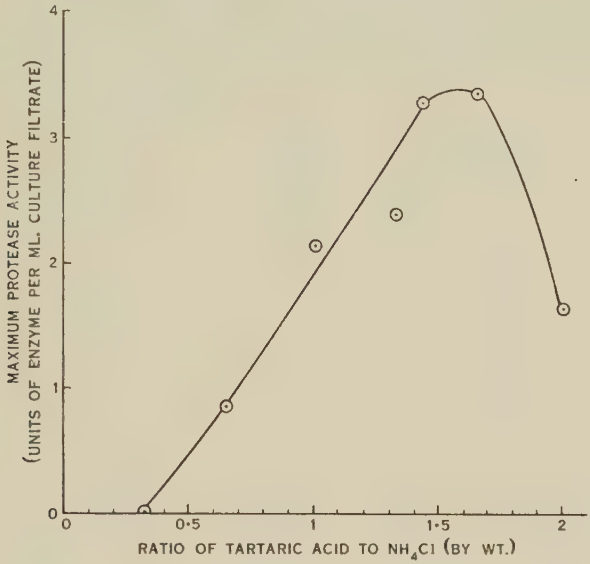

Fig. 1.—Effect of ratio of tartaric acid to ammonia on protease formation (viscometric method).

(*d*) *Comparison of Various Organic Radicals with Tartrate*

It has been found that a number of organic salts can replace tartrate in the medium, with varying degrees of effectiveness for protease production. The optimal concentrations of sucrose and ammonium salts have been determined for media containing salts of malonate, lactate, malate, pyruvate, acetate, formate, fumarate, succinate, citrate, maleate, and oxalate, and it was found that protease production on these media was less than that on the optimal tartrate medium. The activities of the culture filtrates decreased in the order given above.

A more detailed study was made of the changes taking place in media containing tartrate, malonate, lactate, and malate. Table 4 sets out the optimal concentrations of the various components of the media containing these anions as far as protease production is concerned. Sucrose, phosphate, magnesium, and iron were required in the same concentrations as in the tartrate medium.

The changes taking place in these media during growth of the mould are summarized in Table 5. In all media the pH falls initially to values approaching 4.0, then rises above 7.5. With tartrate the minimum pH value is reached

in four days and the pH is restored to 6.0 in six days. However, with the other media tested, although the fall in pH was as rapid, a considerably longer

TABLE 3

EFFECT OF CHANGING SUCROSE AND AMMONIUM TARTRATE CONCENTRATIONS ON THE PRODUCTION OF PROTEASE BY *A. ORYZAE*

Data are provided only for those ammonium tartrate concentrations giving maximum protease activity for each of the sucrose concentrations tested. Incubation temperature 25-27°C.

Sucrose (g./100 ml.)	Ammonium Tartrate (g./100 ml.)	Incubation Time (days)	Viscometric Activity (units/ml.)	Dry Weight Mycelium (g./100 ml. filtrate)
0.1	0.1	7	0.8	0.15
0.5	0.5	6	2.1	0.23
1.0	0.5	6	4.8	0.31
2.0	1.0	6	4.3	0.69
3.0	1.5	6	6.5	1.04
4.0	2.0	6	8.5	1.40
5.0	2.5	7	8.5	1.48
6.0	3.0	8	8.5	1.69
7.0	3.5	9	8.5	1.55
8.0	4.0	10	7.8	1.85
9.0	4.5	10	8.1	2.15
10.0	5.0	12	8.1	2.43

period was required for the pH to rise to 6.0. On the other hand, omission of tartrate or other organic radical resulted in a steady fall of pH to less than

TABLE 4

CONCENTRATIONS OF COMPONENTS OF MEDIA GIVING MAXIMUM YIELDS OF PROTEASE IN THE PRESENCE OF VARIOUS ANIONS

Medium	Molar Concentrations Giving Maximum Protease Activity					
	Organic Anion	NH_4Cl	Sucrose	K_2HPO_4	$MgSO_4 \cdot 7H_2O$	$FeSO_4 \cdot 7H_2O$
Tartrate	0.11	0.21	0.11	0.02	0.002	0.00007
Malonate	0.20	0.09	0.11	0.02	0.002	0.00007
Lactate	0.22	0.05	0.11	0.02	0.002	0.00007
Malate	0.15	0.05	0.11	0.02	0.002	0.00007
No anion	—	0.21	0.11	0.02	0.002	0.00007
No anion but 2% $CaCO_3$* incorporated	—	0.21	0.11	0.02	0.002	0.00007

* $CaCO_3$ was oven-sterilized and added to medium after autoclaving, giving a pH value of 7. The other media were adjusted to pH 6.2.

2.0, and incorporation of calcium carbonate in a medium lacking tartrate resulted in an almost constant pH with little protease production (Table 5).

With all media, the production of mycelium progressed at the same rate, which lends support to the view that the sucrose content of the medium is the chief factor limiting mycelium production.

TABLE 5

EFFECT OF VARIOUS ANIONS ON THE CHARACTERISTICS OF THE CULTURE DURING GROWTH OF *A. ORYZAE** AT 22°C.

Anion in Growth Medium	Maximum Protease (viscometric units)	Weight of Mycelium (g./100 ml. medium) Maximum	Weight of Mycelium (g./100 ml. medium) Final	Maximum Non-Ammonia N (mg./100 ml.)	Minimum Ammonia N (mg./100 ml.)	pH Minimum	pH Time for Return to 7.0 (days)	Final Sucrose Concentration (g./100 ml.)
Tartrate	23 (10 days)	3.0 (6-7 days)	2.0 (12 days)	62 (11 days)	120 (6-7 days)	4.5 (4 days)	8–9	Nil (8 days)
Malonate	18 (11 days)	2.0 (5-7 days)	2.0 (11 days)	54 (11 days)	22 (7-8 days)	4.5 (4 days)	9	Nil (8 days)
Lactate	10 (11 days)	3.0 (7 days)	2.0 (10 days)	23 (11 days)	1 (9 days)	4.5 (5-7 days)	11	0.5 (from 10 days)
Malate	10 (11 days)	3.0 (6-7 days)	2.0 (14 days)	24 (11 days)	1 (10 days)	4.5 (4 days)	11	0.5 (from 9 days)
No anion	0.3 (8 days)	2.0 (6-7 days)	1.0 (10 days)	—	260 (7 days)	1.9 (5 days)	pH constant at 1.9	2.2 (14 days)
No anion; 2% $CaCO_3$	2.0 (14 days)	3.0	—	—	190 (11 days)	5.8 (10 days)	12	Nil (11 days)

* The results in Tables 5 and 6 were obtained with a strain of *A. oryzae*, selected from the original strain, that yielded culture filtrates having higher proteolytic activity than those from the strain used in the earlier experiments.

(*e*) *Inorganic Constituents of the Medium*

For experiments on the inorganic constituents of the medium, the basal medium was modified to contain 2 per cent. ammonium tartrate and 4 per cent. sucrose. The optimal concentration of phosphate in the form of KH_2PO_4 was found to be 0.3 per cent. A seven-day incubation period at 25-27°C. was found to give highest activities for all phosphate concentrations over the range 0.1-0.5 per cent. Figure 2 illustrates the effect of phosphate concentration on the protease activity of the culture.

Media containing varying amounts of magnesium sulphate were prepared for each of a range of phosphate concentrations from 0.1 to 0.5 per cent. The optimum concentration of $MgSO_4.7H_2O$ was found to be independent of the phosphate concentration and was approximately 0.05 per cent. Increasing the magnesium sulphate content above this value led to slower production of the enzyme.

Media that contain potassium in the form of Rochelle salt and potassium phosphate, and chloride as the ammonium salt, do not require the addition of further amounts of potassium chloride.

Of the trace metals, Mn, Co, Ni, Cu, and Mo were slightly inhibitory at a concentration of 0.1 p.p.m., Zn did not inhibit protease production at concentrations up to 1.0 p.p.m., and Fe was found to influence the enzyme production in the manner shown in Figure 3, with optimal production at a concentration of 0.0025 per cent. $FeSO_4.7H_2O$ (5 p.p.m. Fe).

It is almost certain that trace elements are supplied to the mould in the other constituents of the medium. For instance, in large-scale experiments using flat-sided, 2-l. bottles for growing the mould, occasional batches failed, owing to Zn deficiency. It is therefore advisable to add 0.1 p.p.m. of Zn to the medium.

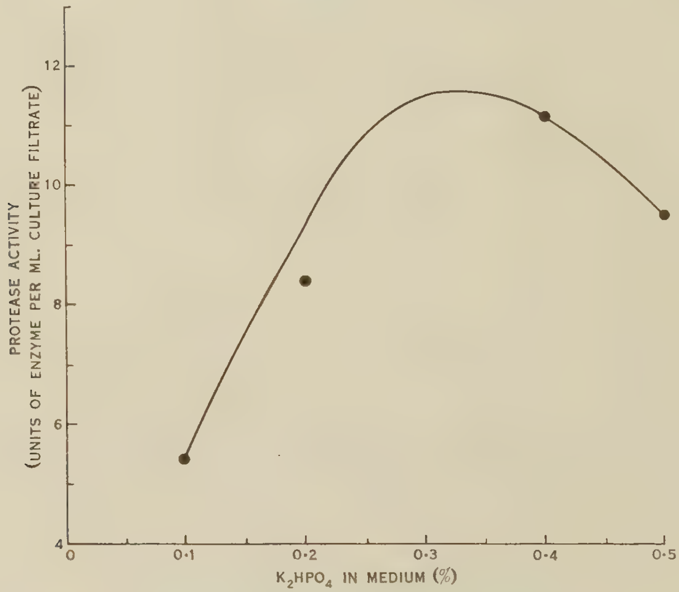

Fig. 2.—Effect of concentration of K_2HPO_4 in the medium on protease formation (viscometric method).

IV. Optimum Conditions for Incubation

Having obtained information regarding the optimum concentration of the medium for production of protease by *A. oryzae* on liquid medium, experiments were continued using flat-sided, 2-l. bottles in place of the 500-ml. conical flasks. The optimum temperature and ratio of volume to surface area were determined for cultures grown in these bottles on the medium described above.

(*a*) *Temperature*

Protease activities were determined by the viscometric and gravimetric methods during incubation at various temperatures. At the higher temperatures the protease developed rapidly but the maximum activity reached was considerably less than in cultures incubated at 18-22°C. (Fig. 4). Thus, at

35°C., the maximum activity of 4.5 viscometric units was produced within four days, whereas at 22°C. the maximum of 27 units required 11 days for its development. Incubation at 18°C. did not provide a higher maximum activity than was obtained at 22°C., and the time for reaching this condition was 21 days. Results obtained by the gravimetric method were similar to the above (Fig. 5).

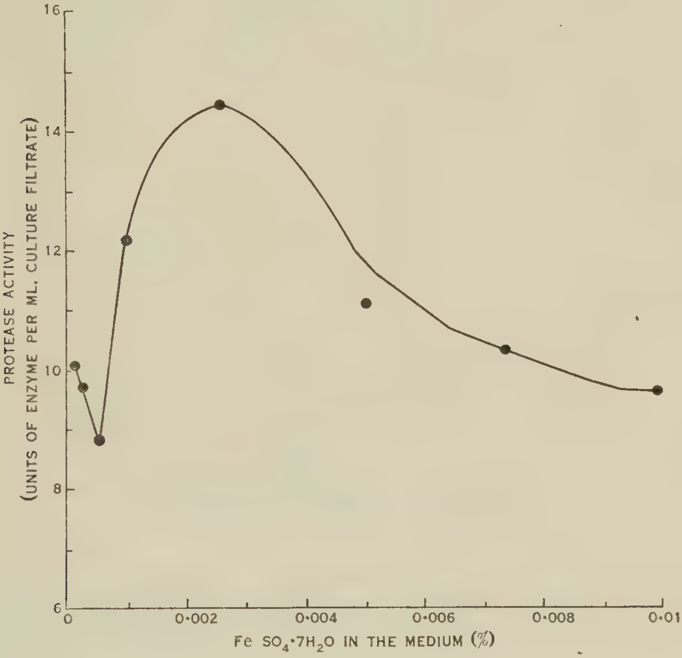

Fig. 3.—Effect of concentration of $FeSO_4.7H_2O$ in the medium on protease formation (viscometric method).

(*b*) *Ratio of Volume to Surface Area*

It has been found that as the ratio of volume to surface area decreased, the activity of the protease produced approached a maximum value. Further decrease of the ratio caused a decrease in the maximum activity reached. Tables 6 and 7 indicate that a volume of 125-225 ml. per bottle, providing a ratio of volume to surface area of 0.52-0.93, gave rise to highest protease activities. As would be expected, increasing the volume of medium per bottle caused a corresponding increase in the time required for the attainment of maximum activity.

During growth of the mould, the volume of medium decreased daily by about 4 ml. per bottle initially, the rate of loss decreasing after about six days to between 1 and 2 ml. per day. At this stage the surface was completely covered with the mycelial mat, which had attained its maximum weight. From Tables 4 and 5 it will be seen that the total recovery of protease per unit

volume of the initial medium was greatest where a volume to surface ratio of 0.93 was used. Using a volume to surface ratio of 0.93, and cultivating the

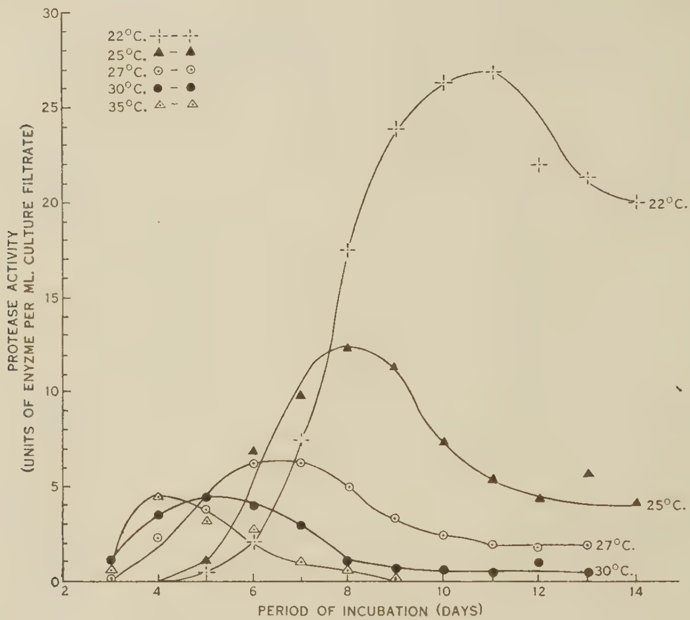

Fig. 4.—Effect of temperature of incubation on protease formation (viscometric method).

Table 6

EFFECT OF RATIO OF VOLUME TO SURFACE AREA ON PROTEASE ACTIVITY (VISCOMETRIC) OF CULTURE FILTRATES*

Volume per 2-l. Bottle (ml.)	Volume (ml.) / Area (cm.^{2})	Maximum Activity (units/ml.)	Time of Incubation at 22°C. (days)	Filtrate Recovered (ml.)	Enzyme Recovered (units/ml. of original medium)
75	0.310	29.0	7	52	20.5
100	0.413	27.5	7	76	20.9
125	0.516	35.0	8	97	27.2
150	0.620	32.7	8	115	25.0
175	0.723	33.7	9	135	26.0
200	0.826	31.5	9	165	26.0
225	0.929	32.5	10	190	27.5
250	1.032	29.0	10	215	25.0
275	1.140	26.0	10	240	22.7
300	1.240	26.2	11	260	22.7
325	1.340	26.0	11	280	22.4
350	1.440	21.0	11	293	17.6
375	1.550	20.4	12	320	17.4

* See footnote to Table 5.

mould at 22°C. for 10 days in 120 bottles, approximately 20 l. of culture filtrate was obtained, with a protease content of 540,000 viscometric units.

V. Final Choice of Medium and Conditions

In the experimental work described earlier, some of the constituents of the medium were tested for optimal concentrations, using a basal medium that differed in some way from the medium found later to give higher protease activities. Further tests were therefore done in which two constituents of the medium were varied and combined in different ways. However, no improvement in the yield of protease could be obtained, and the medium adopted contained 4 g. sucrose, 2.0 g. ammonium tartrate, 0.3 g. K_2HPO_4, 0.05 g. $MgSO_4.7H_2O$, 0.002 g. $FeSO_4.7H_2O$ per 100 ml. of tap water and 0.1 p.p.m. Zn, added as $ZnCl_2$. The optimal initial pH is 6.0-6.5.

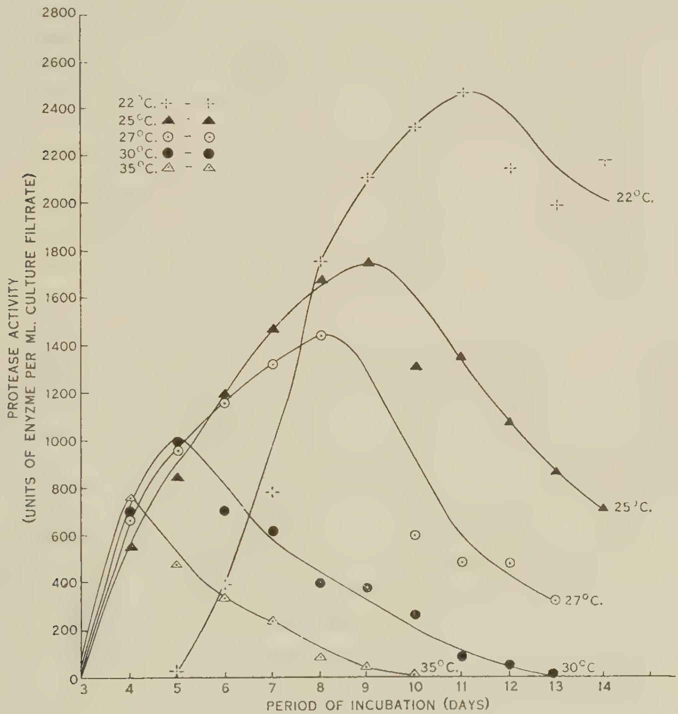

Fig. 5.—Effect of temperature of incubation on protease formation (gravimetric method).

For large-scale enzyme production the ammonium tartrate was replaced by a mixture of sodium potassium tartrate and ammonium chloride. The 2.0 g. of ammonium tartrate in the above medium could be replaced by 3.0 g. of sodium potassium tartrate (Rochelle salt) and 1.1 g. ammonium chloride, and the potassium chloride could then be omitted.

VI. Discussion

During the experimental work described above it has become apparent that there are two requirements for the production of a culture filtrate rich

in proteolytic activity. Firstly, the medium must be capable of supporting an active growth of the mycelial mat responsible for enzyme elaboration, and secondly, its composition and conditions of growth must be such that the required enzyme is produced in substantial amount in the medium. That these are separate considerations is shown, for instance, by the fact that the final protease activity of a culture of *A. oryzae* is not increased by increasing the sucrose content of the medium above 5 per cent., whereas the mycelial weight continues to increase even at double this sucrose concentration.

TABLE 7

EFFECT OF RATIO OF VOLUME TO SURFACE AREA ON PROTEASE ACTIVITY (GRAVIMETRIC) OF CULTURE FILTRATES

Volume per 2-l. Bottle (ml.)	Volume (ml.) / Area ($cm.^2$)	Maximum Activity (units/ml.)	Time of Incubation at 22°C. (days)	Filtrate Recovered (ml.)	Enzyme Recovered (units/ml. of original medium)
150	0.620	3120	8	105	2180
200	0.826	3160	9	155	2450
225	0.929	3120	10	180	2500
250	1.032	3000	11	200	2400
300	1.240	2560	12	250	2130
350	1.440	2520	14	283	2040

It has become generally recognized that moulds are sensitive to low concentrations of trace metals, and the need for magnesium, iron, and zinc in the medium is therefore to be expected. Similarly, the role of phosphate in the metabolism can be conjectured, though not with any degree of certainty. However, the variation of protease production with variation in the concentration of tartrate and with changes in the ratio of tartrate to ammonium salt concentration does not suggest a ready explanation. The fact that omission of the organic radical from the medium leads to a continued fall in the pH of the medium to a very low value, and the observation that no protease is produced in any of the media until the pH has risen from the minimum of 4.0 to approximately the original pH of 6.0 suggests that one role of tartrate is to prevent major changes in the pH of the medium due to assimilation of ammonium ions to form protein. For this to be effective it would be necessary for the tartrate to be metabolized and removed from the medium simultaneously with the ammonium ions. The more rapid return of the pH to the original value in tartrate medium, as compared with media containing other organic radicals, would also support this hypothesis.

A different explanation of the role of tartrate is suggested by the fact that substitution of calcium carbonate for tartrate results in culture filtrate having low protease activities although the pH is buffered at about 6.0. In this medium, growth of the mycelium was excellent. It is apparent, therefore, that tartrate and other organic anions have an important role in the formation or stabilization of the protease complex. Another possible explanation is that

the permeability of the mycelium to enzymes is dependent on the presence of such anions. In the present investigation it has not been possible to establish which explanation is correct.

VII. Acknowledgments

The author gratefully acknowledges the technical assistance given by Miss M. C. Robinson and Miss B. Lyons.

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ENZYMES OF *ASPERGILLUS ORYZAE*

II. THE YIELD OF ENZYMES FROM MUTANTS PRODUCED BY ULTRAVIOLET IRRADIATION

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Summary

Conidia of a strain of *A. oryzae* were irradiated at five different wavelengths, 2480, 2650, 2804, 3132, and 3650 Å. Those germinating after irradiation were isolated by the single spore technique and examined for changes in morphology and for enzyme production on liquid medium. Mutants of the normal strain were produced at all wavelengths, including "lethal mutants."

The 60 stable mutants recovered could be broadly classified into five types. Type *A* comprised strains in which the protease responsible for the reduction in viscosity of gelatin was produced at an earlier stage in growth than either the esterase or the protease acting on the lower molecular weight components of gelatin. Type *B* gave a slightly higher yield, and type *C* a slightly lower yield of proteases than the original strain. Types *D* and *E* developed a much greater mycelial weight and much less enzyme than the normal but the two types were distinguishable on the basis of their appearance.

I. Introduction

Changes in the production of enzymes by sexually reproducing fungi as a result of the induction of mutation by ultraviolet irradiation of the spores of *Neurospora crassa* (Beadle and Tatum 1941) and of *Chaetomium globosum* (McAuley and Ford 1947*a*, 1947*b*) have been demonstrated, and increased production of penicillin has been obtained by selecting mutants of *Penicillium notatum* produced by neutron bombardment (Hanson *et al.* 1946). These results suggested that an increase in the yield of protease obtained from cultures of *A. oryzae* grown on an artificial medium (Maxwell 1951) might be achieved by induced mutation of the spores of this organism.

This paper describes changes in the appearance and enzyme production of *A. oryzae* on irradiation with ultraviolet light at five different wavelengths.

II. Methods

The apparatus used was that described by McAulay and Ford (1947*a*). Since the conidia of *A. oryzae* were found to be much more susceptible to drying than the ascospores of *C. globosum*, no air was passed over the spores

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during irradiation, and moist filter paper strips were held between the quartz glass slides to maintain the humidity close to saturation.

Spores of the normal strain were spread on a thin marked line on a quartz glass slide, using a dissecting microscope. The dosage necessary to give at least a 50 per cent. mortality was determined for wavelengths of 2480, 2650, 2804, 3132, and 3650 Å. After irradiation, groups of 50 to 100 spores were transferred to a petri dish containing beer wort agar, and spread over the surface in 0.5 ml. normal saline with a sterile wire. As each spore germinated it was transferred to a separate agar plate, to be later compared with the original strain in morphology and biochemical reactions. A control was included in each experiment, and for this the spores were spread on the quartz glass slide but were shielded during irradiation. Afterwards they were plated out as described above, and a number of germinating spores, at least equal to those spores taken from the irradiated samples, were transferred to separate plates to check for spontaneous mutation or variation. Cultivation was carried out at 22°C. on the modified Raulin's medium described by Maxwell (1952).

The protease activities were estimated viscometrically by the method of Lennox and Ellis (1945) and gravimetrically by the method of W. G. Crewther (in press). Esterase was estimated by a procedure based on the method of Huggins and Lapides (1947).

TABLE 1

EFFECT OF WAVELENGTH ON MORTALITY AND MUTATION

Wavelength (Å)	Dosage to Give 10 per cent. Mutants (joule/cm.^{2})	Optimal Dosage for Mutant Production (joule/cm.^{2})	Kill at Optimal Dosage (%)	Mutants at Optimal Dosage (per cent. of experimental colonies)
2480	0.045	0.720	75	22
2650	0.017	0.033	80	50
2804	0.021	0.053	77	36
3132	7.5	7.5	40	10
3650	60	60	20	9

III. Experimental and Results

(*a*) *Effect of Wavelength on Mutation*

The results of three or more irradiation experiments at each of the five wavelengths are shown in Table 1. The wavelength 2650 Å was the most effective in producing mutation, closely followed by 2804 Å and 2480 Å. Even at 3132 and 3650 Å, mutants were produced after prolonged irradiation. At 3650 Å, 10 per cent. kill and 10 per cent. of mutants resulted from 4-5 days' continuous irradiation. It was not feasible to increase the dosage at 3132 and 3650 Å to obtain a kill comparable with that obtained at shorter wavelengths, as long exposure resulted in poor germination of the controls.

As beer wort agar is rich in nutrients, it was expected to serve as a satisfactory medium for all mutants. However, a number of "lethal mutants" of the type described by Ford (1948) were observed under the microscope. Conidia of these mutants germinated, but after one or two cell divisions the cell walls ruptured and the cytoplasm was extruded.

It was not practicable to test every colony isolated for the production of enzymes and accordingly the following were selected for study:

(i) Colonies showing differences from the normal strain in rate of growth or in the appearance of the spores or mycelium.

(ii) Colonies developing from slowly germinating conidia or showing irregular development of the mycelium.

(iii) Some apparently normal colonies exceeding in number those included in (i) and (ii).

Before testing the production of enzymes on liquid media, the mutant colonies were subcultured through at least five generations by single spore isolation.

Table 2

DISTRIBUTION OF MUTANT TYPES, EXPRESSED AS A PERCENTAGE OF THE TOTAL ISOLATIONS FOR EACH WAVELENGTH

Wavelength (Å)	Total Isolations	Appearance on Wort Agar—	Normal			Abnormal	
		Appearance on Liquid Medium—	Normal		Abnormal	Normal	Abnormal
		Production of Enzymes on Liquid Medium—	Normal (%)	Abnormal (%)	Abnormal (%)	Normal (%)	Abnormal (%)
2480	44		52	23	2	23	—
2650	54		41	4	9	42	4
2804	80		34	10	16	30	10
3132	8		25	25	0	50	0
3650	17		35	47	0	12	6

The results of testing the 203 cultures from irradiated conidia for growth and protease production on the liquid medium are summarized in Table 2. It will be seen that, after repeated subculturing, a high proportion of form mutants reverted to the original strain. Of those irradiated at wavelengths 2480, 2650, and 3132 Å, only 18-25 per cent. appeared to be stable mutants showing some variation from the normal in enzyme activity on the liquid medium. At the wavelengths 2804 and 3650 Å, the proportions of stable mutants were 36 and 53 per cent. respectively. As will be shown later in this paper, the mutants obtained at 3650 Å were almost all of the same type and were characterized by a slight reduction in yield of proteases. All strains showing abnormal growth on liquid medium produced abnormal yields of enzyme.

Comparison of the 60 mutants with the normal strain in respect of protease and esterase activity and mycelial weight can be drawn from the data given in Table 3.

The strains have been divided into five types (*A* to *E*) as follows:

Type *A* strains include those that produce maximum gelatin viscosity-reducing enzyme earlier than the normal strain. The production of protease acting on the lower molecular weight components of gelatin and esterase production are normal. The maximum viscometric activity is slightly higher than normal but the organism appears normal on both wort agar and on liquid medium. The three mutants of this type were obtained at a wavelength of 2804 Å, but in view of the small number isolated, the figures are not significant.

TABLE 3

ENZYME PRODUCTION BY MUTANTS

		Maximum Enzyme Activity: Viscometric Protease (% of normal activity)		Maximum Enzyme Activity: Gravimetric Protease (% of normal activity)		Maximum Enzyme Activity: Esterase (% of normal activity)		Mean Optimum Incubation Period (days)			Dry Wt. of of Mycelium After 12 days at 22°C. (% of normal wt.)	
Mutant Group	No. of Mutants Tested	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Visco-metric Protease	Gravi-metric Protease	Esterase	Mean	Standard Deviation
A	3	121	4	111	9	120	12	7	10	10	89	4
B	19	124	13	122	8	105	16	10	12	10	86	7
C	27	78	9	82	13	84	11	10	11	10	119	14
D	5	10	8	16	7	25	16	11	12	10	199	60
E	6	25	22	37	25	60	25	11	12	10	147	58

Type *B* comprises strains that appear normal on wort agar but give greatly reduced sporing on liquid medium. The dry weight of mycelium is low, but production of proteolytic enzymes is greater than normal. Esterase production is not changed. With one exception, mutants of this type originated at wavelengths of 2650 or 2804 Å.

Type *C* strains give decreased yields of protease and esterase, the dry weights of mycelium being greater than the normal strain. The majority of mutants obtained at 3650 Å are of this type but they also appeared at all wavelengths.

Types *D* and *E* show the greatest divergence from normal (Table 5). These strains yield less than 50 per cent. of the normal protease production. Type *D* develops up to twice the normal mycelium weight and spores profusely, type *E* produces few or no spores, the mycelium weight being usually higher than the normal. These types predominate at a wavelength of 2804 Å.

The occurrence of specific mutations of *A. oryzae*, similar to that described by Ford (1948) with *Chaetomium*, is not excluded by the above results; but the 203 colonies examined, of which all but 60 reverted to the normal strain on repeated subculture, are too few to give more than an approximate indication of the effect of wavelength on mutation. The wavelengths 2650 and 2804 Å were by far the most lethal for conidia of *A. oryzae* (Table 1). If change in metabolism and enzyme production are accepted as criteria of cell

damage, the results in Table 4 indicate that 2804 Å is the most damaging wavelength of those tested in these experiments. Mutants of types *D* and *E*, and to a lesser extent type *B*, show the greatest differences from the original strain, and these occur from two to four times more frequently after irradiation at 2804 Å than at the other wavelengths used.

TABLE 4

EFFECT OF WAVELENGTH ON TYPE OF MUTANT*

Mutant	Total Number Isolated	Number Isolated at Following Wavelengths 2480 Å	2650 Å	2804 Å	3132 Å	3650 Å
Normal strain *A. oryzae*	122	23	22	27	28	22
Type *A* mutant	3	—	—	3	—	—
Type *B* mutant	19	1	5	13	—	—
Type *C* mutant	27	10	2	5	2	8
Type *D* mutant	5	—	1	4	—	—
Type *E* mutant	6	—	1	4	—	1

* These results refer to strains found to be stable after several sub-cultivations.

(*b*) *Metabolism of Mutants in Relation to Enzyme Production*

Mutants of the same type showed a gradation in enzyme production rather than a clear-cut divergence from the original. For example, gelatin viscosity reduction activity in type *D* mutants ranged from 2 to 21 per cent. of normal. One mutant of each type was grown on the liquid medium for 14 days at 22°C. Changes in the composition of the medium and the production of proteases and esterase were followed daily from the third day onwards. At this stage, no attempt was made to determine the optimum concentrations of the components of the liquid medium for each mutant.

Table 5 summarizes the appearance of the mycelium, utilization of sugar, and variation in pH of the medium during growth. Except for type *A* mutants, there is considerable variation in growth and in the colour of the liquid medium. A bright yellow culture filtrate is normally associated with high enzyme activity, and a very pale or orange filtrate with low activity. The bright orange liquid of type *D* was very slow to filter and contained large crystals of the potassium salt of an unidentified aliphatic organic acid. Sugar utilization by types *A* and *B* was the same as for the normal strain. The more rapid protease formation of type *A* was not correlated with more rapid removal of sugar from the medium. Types *D* and *E* required longer than normal to complete sugar utilization. The estimated 0.5 per cent. reducing sugar present after seven days with type *C* may be a reducing compound synthesized by the mould.

In general, mutants that induced the usual changes in pH of the medium during growth, that is, caused a decrease of pH to approximately 4.5 by the fourth day and restored the pH to the original value by the seventh day, produced enzyme yields that compared favourably with that of the original culture. Types *D* and *E* required longer periods for restoration of the pH and gave low yields of enzyme.

The dry weights of mycelium from cultures on 225 ml. medium are shown in Table 3. Types *A* and *B* both attained maximum mycelial weight two days earlier than the normal strain, and types *C* and *E* one day earlier, while the dry weight of the type *D* mycelium increased to nearly double that of the others. All mutants lost weight when proteases appeared in the medium, and this decrease continued till approximately the twelfth day, after which there was little further change. Autolysis of the mycelium of type *D* mutants, which produced very small amounts of extra-cellular enzymes in the medium, did not liberate more enzymes, nor did disintegration of the mycelium in the Waring Blendor.

TABLE 5

GROWTH AND METABOLISM OF MUTANTS

Mutant	Appearance of Growth on Liquid Medium	Utilization of Sucrose in Liquid Medium During Growth	pH of Liquid Medium During Growth
Normal strain	Even, cinnamon-brown sporing; liquid golden yellow	All sugar disappeared from medium by eighth day	Decreased to 4.5 by fourth day; increased to 6 by seventh day then slowly to 7.5 on thirteenth day
Type *A*	As above	As above	As above
Type *B*	Poor and irregular sporing; liquid bright citron yellow	As above	As above
Type *C*	Even, cinnamon-brown sporing; liquid yellow orange	Fell to 0.5 per cent. on eighth day; then constant	As above
Type *D*	Profuse sporing, bottle green; liquid orange and slightly viscous	All sugar disappeared from medium by tenth day	Decreased to 4.5 by fourth day; remained constant at 6.5-6.8 from eighth day onwards
Type *E*	Few spores; dense white mycelium tending to sink below surface; liquid pale yellow	As above	Decreased to 4.5 by fourth day; increased to 6 by tenth day then slowly to above 7 by fourteenth day

Nitrogen utilization is recorded in Table 6. The nitrogen content of the mycelium varied considerably during growth, decreasing with the age of the mycelium. Types *A*, *B*, and *C* differ little from the normal in the percentage of available nitrogen built into the mycelium, but type *D* used almost twice

TABLE 6

NITROGEN UTILIZATION BY MUTANTS

Mutant	Mycelium Nitrogen: Uptake from Medium (% of N available)	Mycelium Nitrogen: Maximum Loss to Medium (mg.)	N of Medium During Growth: Change in Ammonia N (mg./ml.)	N of Medium During Growth: Change in Soluble Non-Ammonia N (mg./ml.)	Maximum Protease Production: Visco-metric Activity (units/ml.)	Maximum Protease Production: Gravi-metric Activity (units/ml.)	Maximum Esterase Activity (units/ml.)
Normal strain	25	40-50 at tenth day	Minimum at eighth day; rises to 300 at tenth day, then constant	Maximum 100-120 at ninth to eleventh day, then decreases with further incubation	37-38 at tenth day	4600 at eleventh day	428-466 at tenth day
Type *A*	26	40 at seventh day	Minimum 270 at seventh day; rises to 280 at ninth day, then constant	Maximum 130 at seventh day, then constant	41 at seventh day	4600 at tenth day	494 at tenth day
Type *B*	28	80 at tenth day	Minimum 270 at eighth day; rises to 390 at tenth day, then constant	Maximum 130-140 at ninth to tenth day, decrease with further incubation	56 at tenth day	6800 at twelfth day	532 at tenth day
Type *C*	26	40 at tenth day	Minimum 270 at eighth day; rises to 340 at thirteenth day	Maximum 60 at eighth day, then constant	34 at tenth day	4200 at tenth day	361 at tenth day
Type *D*	43	30 at thirteenth day	Falls steadily to 186 at fourteenth day	Maximum 37 at thirteenth to fourteenth day	2 at twelfth day	3400 at thirteenth day	93 at twelfth day
Type *E*	15	30-40 at thirteenth day	Minimum of 325 at ninth day; rises to 391 at fourteenth day	Maximum 50 at eleventh day, then constant	4 at eleventh day	100 at twelfth day	150 at twelfth day

as much and type *E* very much less than the normal strain. Consequently the residual ammonia in the medium was much lower for type *D* and higher than normal for type *E*. The maximum loss of nitrogen from the mycelium to the medium from the time of first appearance of the enzymes corresponded both in time and quantity with the appearance of the proteases and esterase in the solution. It seems probable that this nitrogen loss from the mycelium is due to liberation of enzyme protein, there being simultaneous appearance of soluble non-ammonia nitrogen in the medium.

Type *C* mutants resembled the normal strain when grown on a zinc-deficient medium. Protease and esterase activities were all low and the culture filtrate was more orange than yellow. However, the addition of higher concentrations of zinc to the medium did not improve the yield of enzyme with type *C* mutants.

IV. Discussion

In experiments involving purification of the proteases produced by *A. oryzae* it is of considerable importance to prevent autodigestion of the enzymes during production and handling. The production of highly active protease solution in a relatively short incubation time by type *A* mutants suggests that these strains may find application in the production of protease preparations for purification.

Although no conclusions can be drawn from the limited amount of information concerning specific effects of certain wavelengths, the data presented here indicate that major changes in the production of enzymes by asexual fungi, some of a desirable character, may be induced by ultraviolet irradiation.

V. Acknowledgments

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MALONATE AND CARROT ROOT RESPIRATION

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Summary

Following a review of earlier work with malonate as an enzyme and respiration inhibitor, direct evidence is provided of the existence in carrot root tissue of cytochrome oxidase and succinic dehydrogenase (S.D.). Malonate is clearly effective as an inhibitor of carrot root respiration only at low pH. Its effects at higher pH are, however, fully described and discussed. It is postulated that in this tissue a significant part of the respiration is mediated by enzyme systems not inhibited by malonate, KCN, or CO; that the remainder, whose activity is varied by wounding and aging, and by ionic exchange and uptake, involves an organic acid cycle of the Krebs type. The effects at low pH and low concentration of malonate (0.005-0.02M) may be explained as due to inhibition of succinic dehydrogenase only; under these conditions self reversal of inhibition, and reversal by addition of succinate, are both possible. At higher concentrations (0.04-0.05M) and low pH, malonate is assumed to inhibit not only S.D. but other enzymes concerned in pyruvate oxidation; this explains the lack of self reversal, lack of reversal by added succinate, and the failure to demonstrate accumulation of succinate in poisoned tissue; under these conditions, when inhibition is to the basal level, the R.Q. is high, presumably because pyruvate is diverted to form fermentation products.

I. PREVIOUS WORK

(*a*) *Malonate as an Enzyme Inhibitor*

Malonate has been generally assumed to inhibit succinic dehydrogenase (S.D.) specifically and it has therefore been widely used in the demonstration of the existence of an organic acid cycle in respiration.

Its action on respiration was discovered by Thunberg (1909). Later, Quastel and Wheatley (1925) noted that the rapid dehydrogenation of succinic acid by resting bacteria, in anaerobiosis, was greatly retarded in the presence of malonate. They used a medium buffered at pH 7.4 and succinate was at one-tenth the concentration of malonate. Substituted acids, such as ethyl- or hydroxymalonic acids were inactive.

In 1931 Quastel and Wheatley stressed the importance of malonate in respiration studies and showed that it inhibited the oxidation of succinate by muscle and brain tissue as well as by bacteria. The oxidation of fumarate or malate was not affected by malonate and this provided evidence against the view that fumarate might be oxidized on the succinic enzyme system. More-

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over, malonate did not prevent *p*-phenylenediamine oxidation by tissue, thus the dehydrogenase end of the aerobic oxidation system was taken to be the site of inactivation by malonate.

In 1938 Hopkins, Morgan, and Lutwak Mann provided clear evidence to show that succinic dehydrogenase does not function without intact —SH groups. The enzyme was inactivated by glutathione (GSSG), which oxidizes these groups; after the GSSG had been removed, the activity of the enzyme was restored by the addition of reduced glutathione. It was then found that malonate can protect the enzyme system against the action of GSSG. This observation was confirmed by Potter and Dubois (1943), who also found that malonate protects the enzyme against inactivation by diverse —SH reagents, such as quininoid compounds and heavy metals.

In these experiments (Hopkins, Morgan, and Lutwak Mann 1938) the pH was 7.4; enzymic activity was measured by the rate of reduction of methylene blue or of cytochrome *c*. Malonate inhibition was reversible and the enzyme system, incubated with both GSSG and malonate, was found to have lost none of its original activity after the two inhibitors had been washed away. The protection was afforded by malonate at concentrations much lower than those necessary to inhibit the enzyme activity.

Malonate apparently establishes no special relationships with thiol compounds, so the protection must be indirect. Hopkins, Morgan, and Lutwak Mann (1938), Krebs and Eggleston (1940), and Potter and Dubois (1943) all agree that the inhibition of succinic dehydrogenase by malonate is competitive, as the degree of inhibition depends on the ratio of succinate to malonate present. Potter and Dubois showed that a purified enzyme had a much greater affinity (50/1) for malonate than it had for its normal substrate, succinate. Potter and Elvehjem (1937) have demonstrated inhibition of the succinic enzyme by malonic and oxalic acids and to a lesser extent by adipic and glutaric acids; they agree with the above authors in ascribing the action of these inhibitors to their possession of two —COOH groups, which, for high activity should be in close proximity in the inhibitor molecule. The pH optimum for succinic dehydrogenase is between 7 and 8, and both substrate and inhibitors may be considered to combine with the enzyme as ions. Potter and Dubois have put forward the view that the ions of succinate and malonate compete on the enzyme surface for the same two centres of activity, to which the —COOH groups become attached; the —SH group is supposed to lie between these centres and hence is shielded when the malonate ion is in position. Slater (1949), however, on the basis of evidence that the —SH inhibitors do not act on the dehydrogenase itself, considers that the shielding phenomenon may need re-interpretation.

Malonate is not a specific inhibitor of S.D. as commonly stated. Thus Quastel and Wooldridge (1928), Cook (1930), and Das (1937) all report inhibition of lactic dehydrogenase by malonate. Stare and Baumann (1939) found that malonate at 0.001M caused (in muscle) a greater inhibition of citrate than of succinic oxidation. Das (1937) showed a slight effect of malonate on

the dehydrogenation of malic acid. Pardee and Potter (1949) also found that malonate depresses oxygen uptake by homogenates in the presence of malic acid, but argue that this is because it blocks the removal of the keto acid product. They believe that malonate inhibition of oxidation in the organic acid cycle is due not only to the succinic block but also to the inhibition of oxidation of oxaloacetate in the presence of pyruvate. On the other hand, there is the evidence for the oxidative formation of succinic acid in muscle brei (Krebs and Eggleston 1940) and plant tissues (Laties 1949*b*) when malonate is present. When such succinic accumulation occurs the block at oxaloacetate must be only of minor importance.

Finally, both Evans, Vennesland, and Slotin (1943) and Liebecq and Peters (1949) agree that malonate inhibits the enzymic decarboxylation of oxaloacetate to pyruvic acid, although the latter authors think that this may be of not much importance in some tissues, in which spontaneous decarboxylation is active. It seems therefore quite likely, at least in experiments with the higher concentrations of malonate, and especially where succinate accumulation cannot be demonstrated, that effects on respiration cannot safely be ascribed simply to the effect on succinic dehydrogenase. The other enzymes shown to be affected by malonate are all concerned with the Krebs cycle. Pardee and Potter (1949), however, ascribe the action of higher concentrations of malonate on oxaloacetate oxidation to the formation of a malonate complex with magnesium. As this is a component of other enzyme systems (e.g. in glycolysis) a malonate effect on such systems cannot be ruled out.

(*b*) *Malonate and the Organic Acid Cycle*

When Gözsy and Szent-Györgyi (1934) published the dicarboxylic acid-hydrogen transport theory for muscle tissue, they pointed out the value of malonate as a specific inhibitor and it has subsequently proved to be a most useful reagent in the study of the organic acid cycle. Thus Krebs (1943) states that the most important experimental observation in support of the existence of this cycle is that succinate can be formed oxidatively from fumarate or oxaloacetate when its own oxidation and its reductive formation from these acids is blocked by malonate. This observation has often been made and from it Krebs argues that during respiration there is a cycle of oxidation in which the dicarboxylic acids arise periodically (see also Green, Loomis, and Auerbach 1948).

Malonate at pH 7.4 does inhibit the oxygen uptake by minced animal tissue and simultaneously succinate accumulates (e.g. Krebs and Eggleston 1940). The picture is not quite so clear, however, when we consider work with intact tissues rather than with cell-free extracts or minced tissue. In 1936 Greville observed that 0.01M malonate at pH 7 was strongly inhibitory to the respiration of minced pigeon breast muscle. But as the addition of physiological concentrations of calcium proved equally inhibitory, he concluded that the tissue had been rendered "unphysiological" by mincing. Accordingly, he repeated his experiments, this time with thin rat diaphragm, damaged very little by

handling. Calcium did not affect the rate of respiration and malonate (0.01M) was only slightly effective. Even at a concentration of 0.05M, inhibition by malonate was incomplete and reached its steady value only after 100 minutes. If the diaphragm were severely damaged by being cut into small pieces, the low malonate concentration (0.01M) produced an immediate response and calcium was likewise inhibitory.

This increased sensitivity of damaged tissue for malonate was also observed by Boyland and Boyland (1936) when investigating tumour respiration. The responses to succinate and fumarate were similarly enhanced by wounding. Weil Malherbe (1937) also found that "the succinic dehydrogenase system seems to be protected from malonate in the structurally intact tissue." Malonate at 0.04M and pH 7 (substrate ketoglutarate) reduced the oxygen consumption of tissue slices by only 50 per cent.; the R.Q. was not affected and succinate did not accumulate. Minced tissue more closely resembled the pure enzyme system in which much weaker malonate, also at pH 7, completely stopped the enzyme action and brought about accumulation of succinate. He considered that "in tissue the effects are complex and cannot all be attributed to a specific action of malonate." However, as we shall see, the anomalous results with tissue are probably due to the use of too high a pH in the medium. The pH optimum for the enzyme reaction is usually quoted as being between 7 and 8; biochemical work on the system is always carried out over this range and experiments with animal tissue generally require alkaline or neutral pH.

Succinic dehydrogenase is an insoluble enzyme and evidence is accumulating to show that it is attached to particles of mitochondrial size (e.g. Chantrenne 1943). It is comparatively easy to obtain a stable suspension from animal cells. In plants, succinic acid is commonly present in small amounts and the acid is certainly metabolized (e.g. Turner and Hanly 1949); there are strong *a priori* reasons for supposing that the enzyme is widely distributed in plant issues. Okunuki (1939) reported its presence in pollen, and Goddard (1944) showed that wheat embryos and their dispersions had a low S.D. activity. Berger and Avery (1943, 1944), however, could not demonstrate its presence in oat coleoptiles, in which other dehydrogenases were active. Bonner and Wildman (1946) showed that the fraction of spinach respiration that is malonate-sensitive is rapidly lost when the tissue is frozen and then thawed and they take this to be evidence for the existence of the enzyme, but in a labile state. This lability, also noted by Goddard (1944), may account for the failure to discover the enzyme in some plant tissues.

In plants, therefore, malonate has been mainly applied to whole plant tissue and in the early work it was usually reported to be ineffective, or even to stimulate respiration. Burris and Wilson (1939) studied the oxygen uptake by *Rhizobium* in phosphate buffer, plus substrate, at pH 6.5. Under such conditions the greater part of the respiration was cyanide-sensitive and cytochrome activity was shown, spectroscopically. It was therefore expected that malonate would prove an effective inhibitor; however, at 0.04M it caused "slow growers"

to increase their rate of oxygen uptake, while with "fast growers" the response to malonate varied from 82 per cent. stimulation to 15 per cent. depression of the oxygen uptake. Results were different in Thunberg experiments. Decolorization of methylene blue, with either glucose or succinate as substrate, was inhibited 40-50 per cent. by 0.04M malonate and inactivation was still evident at 0.01M. The authors took the view that stimulation of respiration might have been due to the malonate combining with copper ions, which inhibit succinic dehydrogenase. They also suggested, on very scanty evidence, that malonate at some concentrations was a good substrate for *Rhizobium*. Again, in 1943, Albaum and Eichel showed that malonate at pH 6 actually stimulated the respiration and also the growth rate of oat coleoptiles by as much as 27 per cent. Malonate thus resembled other organic acids, such as malic, pyruvic, and succinic acids, which are regarded as substrates rather than as inhibitors of respiration. Henderson and Stauffer (1944) also obtained little, if any, inhibition of tomato root respiration by malonate at pH 5.2-5.8 and occasionally they obtained stimulation.

Machlis (1944), using barley root tissue, was the first to obtain consistent inhibition of plant respiration by malonate; he was also the first in this field to use a medium of low pH. It has since been shown that as long as the malonate is applied at low pH (*c.* 4-4.5) is will rapidly inhibit a large part of the respiration in several different tissues, viz. spinach leaf (Bonner and Wildman 1946), carrot root (Turner and Hanly 1947), oat coleoptile (Bonner 1948), barley root (Machlis 1944; Laties 1949*a*), and rhubarb (Morrison 1950). At pH higher than 4.5 the inhibition is less marked and temporary, while at neutral pH there may be stimulation. It is part of the purpose of the present paper to discuss this pH effect but before doing so we shall conclude this review by referring to two other features of importance.

If malonate inhibits respiration through its competitive action on succinic dehydrogenase it should be possible to obtain reversal of the inhibition by the addition of succinic acid or of other acids of the Krebs cycle. Machlis (1944) could obtain no such reversal, although Laties (1949*a*), working also with barley root tissue, did so. Bonner and Wildman (1946) and Bonner (1948) agree with Laties in obtaining reversal (by succinate), but only under certain conditions. Laties (1949*b*) and also Bonner (1948) have provided clear evidence that succinate accumulates in tissues treated with malonate, and this is regarded as strong evidence that an organic acid cycle exists in some plant tissues. Their work will be discussed at greater length after the presentation of our own results.

In all the work so far dealt with, the assumption has been made, and sometimes supported by experimental evidence, that succinic dehydrogenase is coupled with cytochrome oxidase. Bonner (1948), however, has now obtained malonate inhibition in spinach leaf tissue, for which he presents reasons for believing that the terminal oxidase is polyphenol oxidase. Rosenberg and Ducet (1949) and Stenlid (1949) have thrown doubt on this latter conclusion and further work along these lines is obviously desirable.

II. Cytochrome Oxidase and Succinic Dehydrogenase in Carrot Tissue

Both these enzymes have been prepared from carrot root phloem parenchyma. Details will not be presented here† but the results merit brief report. A crude preparation was obtained by maceration of the tissue in a Waring Blendor in M/15 phosphate buffer, pH 7.4, followed by centrifugation of a muslin filtrate of the blend; it oxidized cytochrome *c* in the presence of *p*-phenylenediamine, the optimum pH being between 7.2-7.8; the Michaelis constant varied from 3.5 to 4.8×10^{-6}M, of the same order as that calculated by Goddard (1944) for the cytochrome oxidase of wheat embryo. The enzyme was inhibited by KCN (10^{-3}M) and there was light-reversible inhibition by carbon monoxide. The same extract would not oxidize succinic acid but an active succinic dehydrogenase was prepared by similar methods when greater care was taken to conduct all preparatory operations at 5°C. This extract brought about oxidation of succinic acid in the presence of either methylene blue or cytochrome *c*. The optimum pH for the reaction was 7.3. The enzyme activity between pH 6.2 and 8.3 was partially inhibited by 0.01M malonate and this inhibition was shown to be competitive. These facts therefore provide justification in what follows for assuming that malonate inhibition of respiration is, in part at least, due to the inhibition of succinic dehydrogenase; also that the succinate-dehydrogenase of the carrot root is inhibited *in vitro* by malonate at high pH.

III. Effect of Malonate on the Respiration of Carrot Root Tissue

(*a*) *Experimental Methods and Material*

The plant material was secondary phloem parenchyma from the storage roots of carrot (Danvers Half-long variety). For measurement of respiration, discs 1 mm. thick, 0.8 cm. diameter, were cut by microtome and cork borer and aerated in distilled water for periods up to 400 hours. Oxygen uptake and carbon dioxide output were measured by the two-vessel method of Warburg, values for the R.Q. in media of pH greater than 6 being checked by acid tip. The methods were exactly as reported in a previous paper (Turner and Hanly 1949). As solutions of buffer salts may themselves have marked effects on the respiration rate, the tissue was usually suspended in distilled water and the malonate solution added later from the side-arm. The pH of the medium was measured at the start and finish of each experiment. For some of the work at low pH the tissue was maintained throughout in M/15 phosphate buffer.

Previously a distinction has been made between two types of carrots used in these experiments; type *A*, the respiratory rate of which falls gradually after 80-100 hours of aging in water, to a value below $Q_{O_2}^{FW}$* = 100; and type *B* with a much higher respiration rate remaining above $Q_{O_2}^{FW} = 160$. It is believed that

† This work will be separately reported by K. S. Rowan.

* $Q_{O_2}^{FW}$ = cu. mm. of oxygen consumed per g. fresh weight of tissue per hour.

the differences between carrot root tissue of types *A* and *B* are quantitative rather than qualitative and results with both types are treated together.

In preliminary experiments it was found that the effects of malonate may vary with the age (hours from cutting) of the tissue slice. Hence, usually one large batch of discs were cut and washed continually in aerated distilled water and each experiment (three or four treatments and controls) was repeated at intervals of several days with slices taken from this batch.

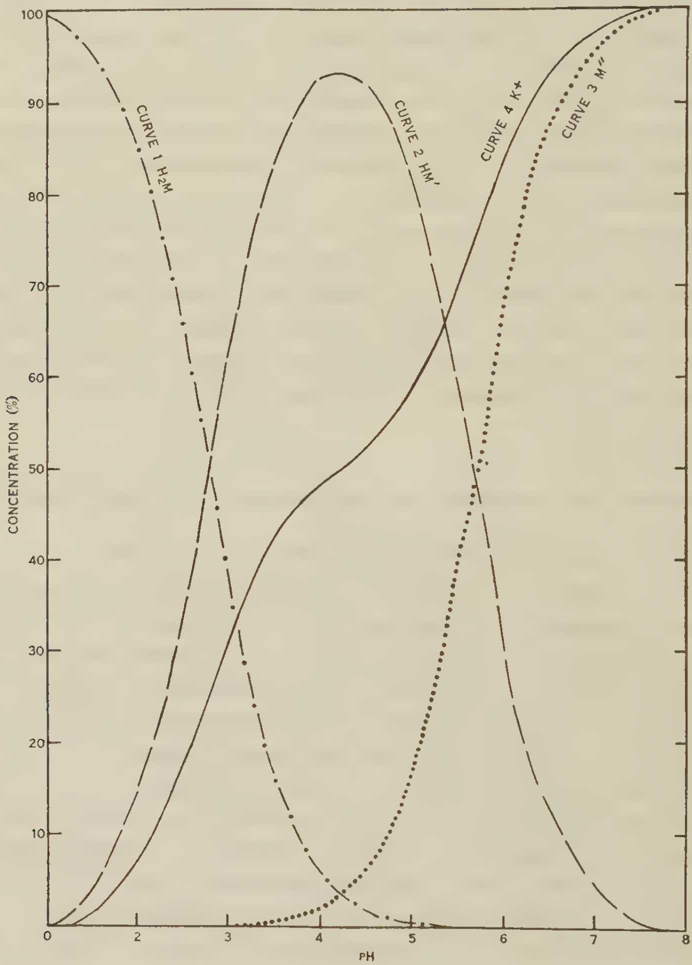

Fig. 1.—Dissociation curves for potassium malonate.

1. Percentage concentration of undissociated malonic acid.
2. Percentage concentration of monobasic malonate ion.
3. Percentage concentration of dibasic malonate ion.
4. Half percentage concentration of potassium ion (thus at pH 4, $K^+ = 96.2$ per cent. of the total molarity of the solution).

Like succinic acid, malonic acid is a weak dibasic acid and in view of the results already obtained for succinate (Turner and Hanly 1949) it was con-

sidered important to test the effect of malonate over a range of pH. The relative concentrations of undissociated malonic acid (pK_1 2.5; pK_2 5.7) and of its ions at increasing pH have been calculated and are presented in Figure 1. We also include the curve for potassium ion concentration over the same range, since the pH was adjusted by adding potassium hydroxide to the acid solution. These curves have been used to determine the molecular and ionic concentrations to be quoted.

(*b*) *Respiratory Effects of Malonate applied at pH* 4-4.5

(i) *Oxygen Uptake* (*pH* 4-4.5)

As already reported (Turner and Hanly 1947) we have found that malonate causes a clear-cut and continuous depression of carrot root respiration only when it is applied at or near pH 4. The effect is illustrated in Figures 2, 4, 6, 7, 8, 9, 11, 12, and 13, and in Table 3.

In another paper (Turner and Hanly 1949) it has been shown that phosphate buffers at pH 4.5 neither stimulate nor depress the rate of the oxygen uptake in carrot root tissue; succinate at pH 4 brings about maximal stimulation and neutral phosphate buffers may also stimulate carrot root respiration. In our experiments we have only obtained *inhibition* of respiration at *low* pH when inhibitors such as malonate or cyanide are also present in the external solution and we shall therefore take it that these inhibitions are not due to the low pH *per se*, but to the presence of the inhibitor. The effect of malonate at pH 4.5 is the same whether the acid is supplied in water or in M/15 KH_2PO_4 phosphate buffer.

There is some variation in the sensitivity to malonate for slices of different age and for tissue from different batches. As a result of numerous experiments over three years, we may generalize as follows. Malonate at pH 4-4.5, from 0.005M to 0.01M, causes only slight depression of the oxygen uptake; minimal values are reached within 20-30 minutes, after which recovery takes place to the normal $Q_{O_2}^{FW}$ or above it. Malonate at 0.03-0.05M depresses the $Q_{O_2}^{FW}$ to a value that is reached about 30 minutes after application of the inhibitor and is maintained steady for at least four hours subsequently. The inhibition curves for 0.04M and 0.05M malonate are substantially similar to that for 0.03M, although the steady values may be slightly lower. The mean steady value given by 0.05M malonate (which we regard as a critical concentration) in 29 experiments with widely different batches of carrot tissue was $Q_{O_2}^{FW} = 37.3$, S.E. 2.02 (total $Q_{O_2}^{FW}$ of controls 72-230).

When the malonate concentration is increased from 0.05M towards 0.1M the $Q_{O_2}^{FW}$ is suppressed significantly below this value, and moreover, the inhibition time curve now shows a continuous downward trend. Such solutions obviously damage the cells irreversibly; they lose turgor and some of their contents, the solution bathing them becoming yellowish and opalescent. The effects summarized above are illustrated in the results for a single experiment in which

nine comparable sets of slices were subjected to different malonate concentrations, the pH being maintained at 4.5 by phosphate buffer (Fig. 2).

These results are similar to those obtained by other workers with different tissues. The concentration of malonate required to produce marked inhibition of oxygen uptake is near 0.05M for carrot root, barley root (Machlis 1944; Laties 1949*a*), spinach leaf (Bonner and Wildman 1946), and for starved *Avena* coleoptiles (Bonner 1948). Normal *Avena* coleoptiles require near 0.2M malonate for 90 per cent. inhibition. In all these experiments inhibition was greatest at pH 4-4.5. So far, only in *Arum* spadix tissue has respiration been shown to be unaffected by malonate at low pH (James and Beevers 1950).

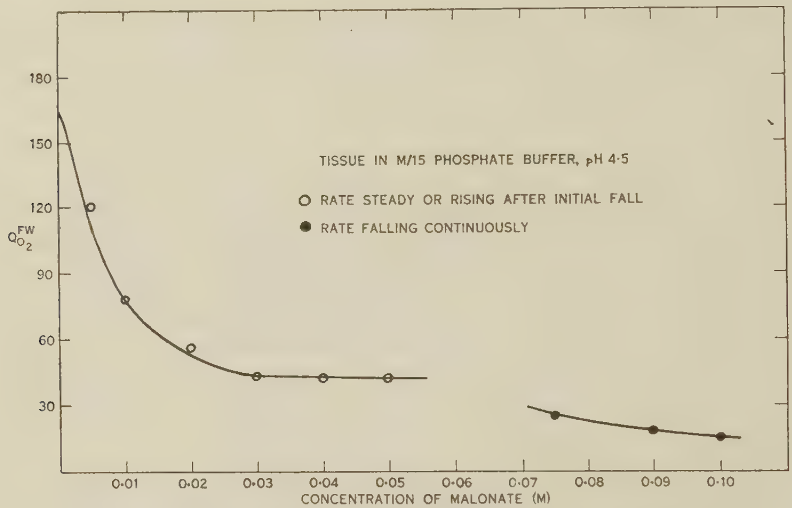

Fig. 2.—Relation between respiration rate (two hours after addition of malonate) and malonate concentration, pH 4.5. All slices of same age and in M/15 phosphate buffer. Initial rate in buffer (mean), $Q_{O_2}^{FW} = 165$.

In none of the papers quoted is it stated that inhibition of oxygen uptake is complete, even with high concentrations of malonate. In our own experiments the results strongly suggest that there is a basal malonate-resistant respiration system and that variations in total Q_{O_2} are mainly due to variations in the malonate-sensitive system. If this is so, then as Commoner (1940) has pointed out, comparisons of percentage inhibition have little meaning. In earlier work (Robertson and Turner 1945) we have postulated the existence of a cyanide-resistant basal respiration and more recently, using carrot roots of the same stock as those treated with malonate, we have found that cyanide at 5×10^{-4}M brings down the $Q_{O_2}^{FW}$ to a basal rate that is not decreased when the cyanide concentration is increased to 10^{-3}M. The effect of cyanide therefore resembles that due to malonate, with one possibly important difference. The basal respiration in malonate remains steady with time, whereas that in cyanide shows usually a very slow but significant, almost linear, fall with time.

In his discussion, Commoner (1940) has adopted the hypothesis that for any tissue the cyanide-stable respiration (b) remains at a steady value while the cyanide-sensitive respiration (x) varies widely. He therefore plots y (total R) against x, obtaining a straight line, at 45° slope, cutting the ordinate at the value of b.

In our experiments with both cyanide and malonate there was a suggestion that the level of the basal respiration varied slightly with the age of the slices and with the rate of total respiration. The data for the group of experiments in which both inhibitors were used have therefore been analysed. We use the following symbols:

TR = total respiration ($Q^{FW}_{O_2}$),
BR_c = basal rate (extrapolated to zero time) in cyanide ($Q^{FW}_{O_2}$),
BR_m = basal rate (mean) in malonate ($Q^{FW}_{O_2}$),
A = age of tissue in hours from cutting.

For 29 experiments with malonate the simple correlation coefficients were: BR_m/TR, $r_1 = 0.427$; BR_m/A, $r_2 = 0.169$; TR/A, $r_3 = 0.152$. Of these, only r_1 is significant and at the 2 per cent. level. Calculation of partial correlation coefficients shows also that there is no significant linear correlation between BR_m and A (eliminating TR) and that for BR_m and TR (eliminating A) the coefficient is 0.4123, significant at the 5 per cent. level. Thus the effect of equalizing for age is to reduce the significance of the correlation between BR_m and TR to the 5 per cent. level, from the 2 per cent. level. Experiments with carrot tissue over several years have shown that there is a relationship between age of slice and TR, but it is complex, as both young and old slices may have a lower rate of respiration than slices of intermediate age. For the experiments under review, however, all that is established is that there is a linear relationship between BR_m and TR, the regression equation being

$$BR_m = 0.109 \times TR + 21.44.$$

Thus an increase in total respiration is accompanied by a small but significant increase in the rate of the basal respiration.

For comparison of the effects of malonate and cyanide we have plotted the regression of BR_m and of BR_c on TR (Fig. 3). The regression coefficients were 0.109 and 0.152 respectively and these are significant, but not significantly different from each other. Therefore, a common regression coefficient was calculated for the 29 malonate and the 53 cyanide experiments, the regression lines being:

$$BR_m = 37.3 + 0.131\,(TR - \overline{TR});\ \overline{TR} = 146.0$$

$$BR_c = 49.7 + 0.131\,(TR - \overline{TR});\ \overline{TR} = 126.3.$$

Thus both for cyanide and malonate the basal respiration shows the same increase with increased TR. The means for BR_m and BR_c (37.3 and 49.7) are significantly different and this suggests that the fraction of the respiration insensitive to cyanide is quantitatively different from that insensitive to malonate. This by no means follows, however. There is considerable difficulty in decid-

ing what figure to use for BR_c as the rate in cyanide continually declines,* whereas that in malonate is steady after two hours. Hence our figures for BR_c, obtained by extrapolation to zero time in cyanide, are not strictly com-

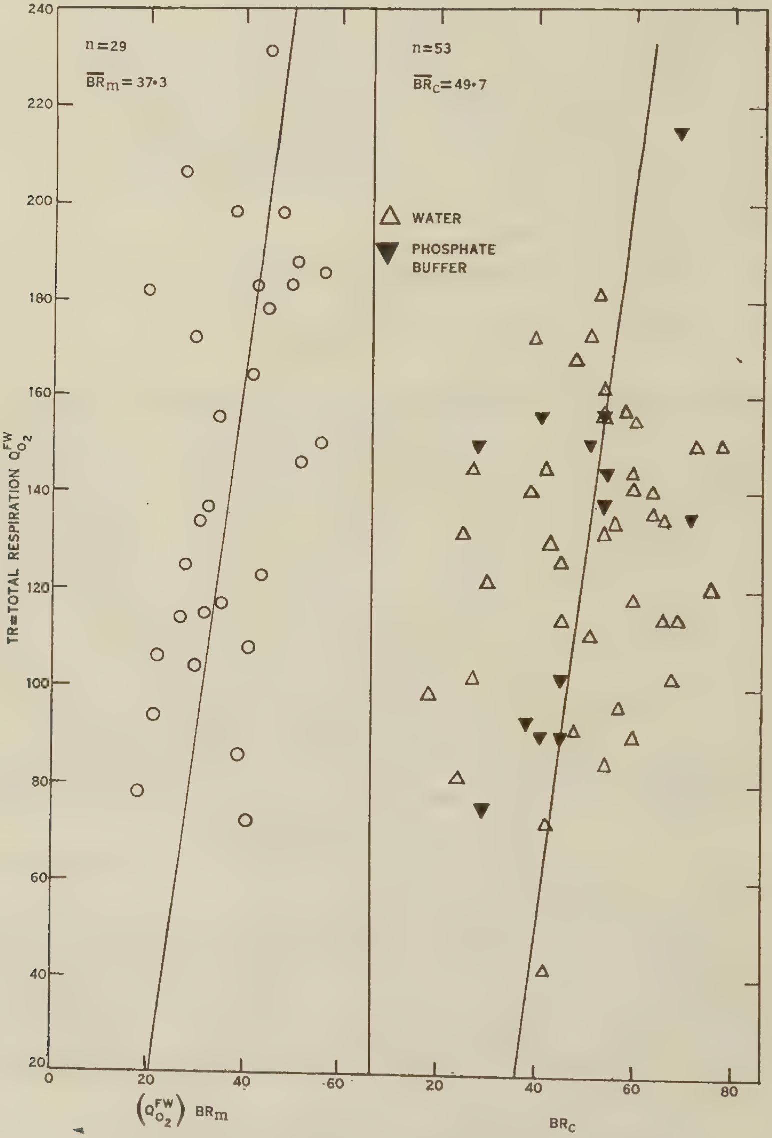

Fig. 3.—Regression of basal respiration in malonate (BR_m) and in cyanide (BR_c) on total respiration.

parable with those for malonate. By arbitrarily choosing a time at which to determine BR_c (say four hours after addition of cyanide) it would undoubtedly

* This feature is not brought out in Figure 4, for which the basal respiration in cyanide is atypically steady.

be possible to obtain equality of $\overline{BR}_c$ and $\overline{BR}_m$; for obvious reasons, this has not been done. It must also be borne in mind that, as both inhibitors form dissociable complexes with their respective enzymes, complete inhibition of the sensitive fractions of the respiration will not be obtained, inhibition being "only as complete as is required by the dissociation law" (Warburg 1949).

(ii) *Respiratory Quotients in Malonate* (0.05*M*; *pH* 4)

Respiratory quotients were measured in some experiments by the two-vessel method of Warburg. Table 1 gives figures and Figure 4 presents the graph for a typical experiment. An initial gush of CO_2 was always obtained on tipping malonate solutions at pH 4 into distilled water surrounding the tissue slices. We have previously (Turner and Hanly 1949) explained this as due to the increased acidity of the tissue medium expelling CO_2 from bicarbonate present in the medium and tissue. This initial gush (first 20 mm. reading) was not included in calculations of the mean R.Q. The data are consistent; while the malonate at pH 4 rapidly reduces the rate of oxygen uptake, the rate of CO_2 output declines more slowly and the R.Q. rises rapidly to about 3.

TABLE 1

$Q_{O_2}^{FW}$ AND R.Q. OF CARROT ROOT TISSUE IN CONTACT WITH 0.05M MALONATE AT pH 4

Carrots	Time from Cutting (hr.)	pH of Malonate	$Q_{O_2}^{FW}$ in Dist. Water Prior to Malonate	$Q_{O_2}^{FW}$ in Malonate	R.Q. Range in Malonate	Mean R.Q. in Malonate
Type *A*	1	4.2	90	30	2.8-3.6	3.0
	20	4.0	132	36	2.9-3.5	3.2
	20	4.2	183	60	2.6-2.9	2.7
	20	4.2	183	48	—	2.7
	20	4.1	204	45	2.6-3.6	3.2
Type *B*	48	4.3	198	42	3.0-3.8	3.3
	70	4.2	—	36	1.6-3.6	2.6

Figure 4 illustrates the results of an experiment in which the respiratory quotients of tissue in malonate and in cyanide were measured concurrently. The cyanide solution had a pH of about 5.5 and did not produce the initial gush of CO_2 always observed with the more acid malonate solutions. The mean quotients for both inhibited tissues were high (*c.* 2.5) and not significantly different. Cyanide inhibition is known to be accompanied by aerobic fermentation in some plant tissues and we presume the same to occur with malonate. The presence of alcohol has been detected in malonate-poisoned tissue, but so far no quantitative estimates have been made.

These results may be compared with those of other workers. Machlis (1944) publishes one curve showing marked inhibition of both oxygen uptake and CO_2 output by malonate in barley roots, but the final R.Q. in malonate (0.05M) is nevertheless 1.6 as against 1.0 for the control and 2 for cyanide (0.005M). Malonate at 0.01M gave about 50 per cent. inhibition and an R.Q. of 1.14. On the other hand, Bonner and Wildman (1946) (one experiment

quoted) found that malonate inhibited the oxygen uptake of spinach leaf by 90 per cent. and CO_2 evolution by approximately the same amount. R.Q. data are not available for *Avena*. Laties (1949*a*) stated that in his work with barley roots the R.Q. in malonate was very close to the control. However, the only figures quoted show a 24 per cent. decrease of Q_{O_2} and a 5 per cent. decrease of Q_{CO_2}, the R.Q. being markedly changed from 0.82 to 1.03 (with fumarate and malonate added the R.Q. returned to 0.79).

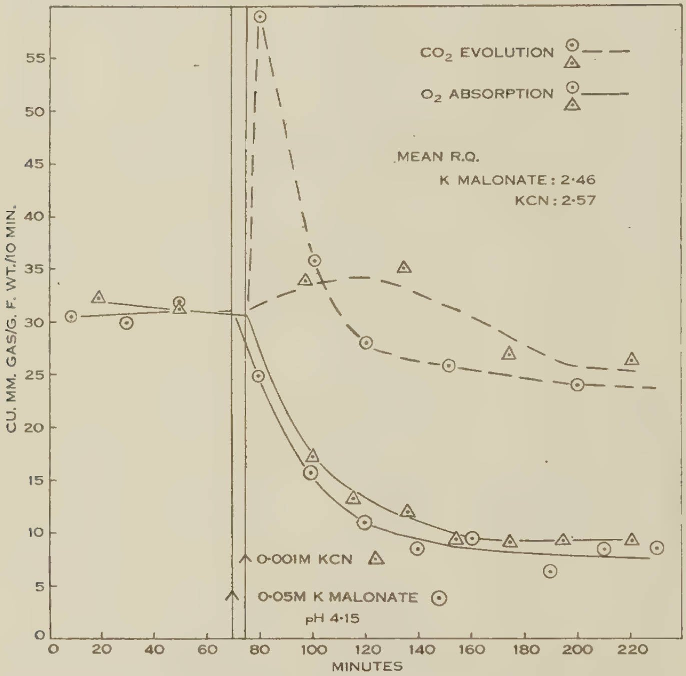

Fig. 4.—Comparison of rates of gas exchange in 0.05M malonate, pH 4.15 and in 0.001M KCN. Mean R.Q. calculated with omission of first two readings. Tissue used 44 hours after cutting.

(iii) *Inhibition of Salt Respiration* (*pH* 4-4.5)

It is known that when carrot root respiration is stimulated by neutral salts (e.g. KCl), the respiration is depressed to the "basal" level by cyanide (Robertson and Turner 1945) and by carbon monoxide (Weeks and Robertson 1950). Malonate at 0.05M and pH 4 has the same effect on the salt-stimulated respiration as have the other two inhibitors. The level of the basal respiration is not altered in the presence of KCl although, of course, the percentage inhibition of the total respiration is markedly changed.

(iv) *Reversibility of the Malonate Inhibition* (*pH* 4-4.5)

Self reversal.—When carrot root slices are supplied with malonate at pH 4 at a concentration of 0.005-0.01M, the inhibition is not complete and recovery towards (or even above) the normal rate takes place in the presence of the inhibitor (Fig. 5).

Slow recovery in weak solutions of malonate is suggested by Figure 6 of Laties's (1949*a*) work on barley root; it is also shown in our own experiments when stronger malonate solutions are applied at pH 5 or 6 (see below).

On removal of malonate in the external solution.—The inhibitory effect of malonate on succinic dehydrogenase *in vitro* is reversible if the enzyme is washed with water (Hopkins, Morgan, and Lutwak Mann 1938). The inhibition of the respiration in carrot tissue is also removed if the applied malonate solution is replaced with water.

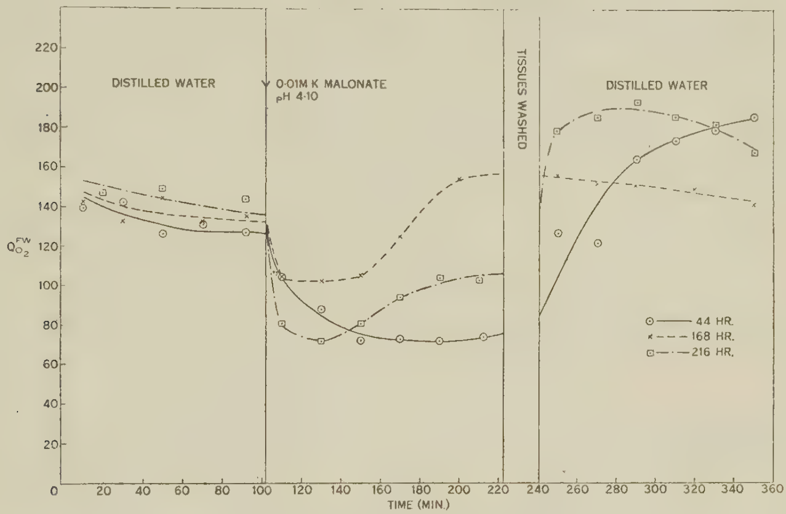

Fig. 5.—Self-recovery from partial inhibition of respiration by 0.01M malonate, pH 4.1, and the effects of replacing this by water. Tissue used 44, 168, 216 hours after cutting.

Reversibility was first tested in those experiments in which malonate at concentrations ranging from 0.05M to 0.03M brought the respiration down to the steady basal rate. Each set of slices was treated with the malonate solution for two hours. The tissues were then quickly rinsed with four successive changes of distilled water and the drifts of the rates of oxygen uptake were subsequently followed in distilled water. Results are graphed in Figures 6 and 7. It is clear that recovery takes place; the rate of recovery is a function of the age of the tissue slice (Fig. 6) and it is shown that, except with aged discs (with 0.05M malonate), the final rate attained is greater than that before inhibition.

During the inhibition of respiration the R.Q. is high (*c*. 3) but after washing it falls rapidly. It is, however, important to note that even after the respiration has risen to or above its normal value, the R.Q. remains near 1.3.

The self recovery noted above is accelerated and its extent usually increased if the weak malonate solution is replaced by water (Fig. 5).

Reversal by addition of succinic acid; competitive inhibition.—If malonate does not affect glycolysis and acts by inhibiting one step only of the Krebs cycle, and if the inhibition is competitive, one might expect to obtain reversal of inhibition by adding succinic acid or indeed other acids that give rise to this in the cycle, e.g. fumaric acid. In several early experiments (Turner and Hanly 1947), mostly with 0.05M malonate, we were not able to obtain such reversal or any indication that the inhibition was competitive. Thus the rate of oxygen uptake of malonate-poisoned tissue was not affected by the presence of succinic or fumaric acids, even when these were present in concentrations up to five times that of the malonate. This obtained whether these acids were added to the tissue before, with, or after the malonate. These negative results of reversal experiments were in accordance with those of Machlis (1944) for barley roots, but are in disagreement with the more recent findings of Bonner and Wildman (1946), Bonner (1948), and Laties (1949*a*). Late publication of the present paper has allowed a fuller investigation of this aspect of the problem.

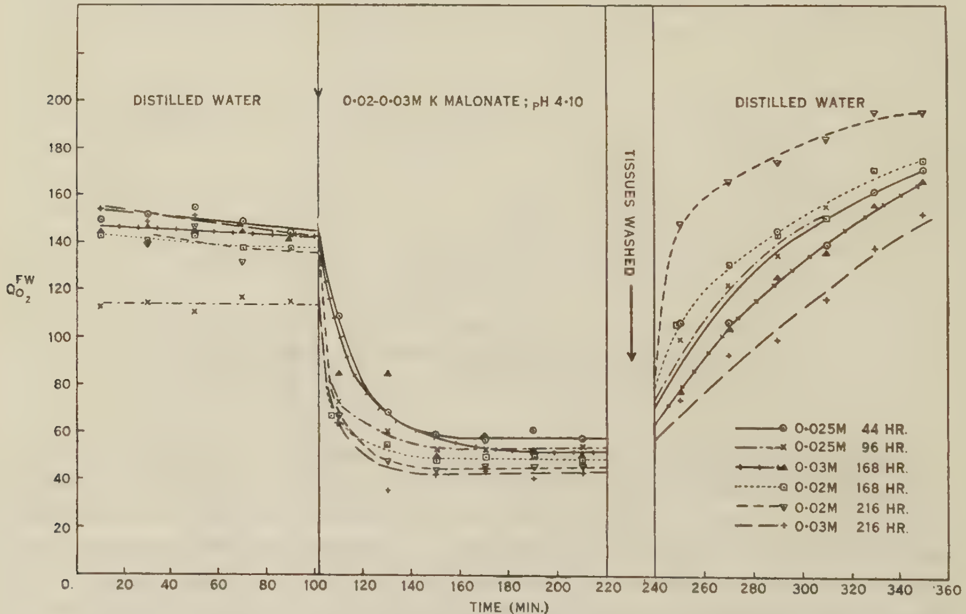

Fig. 6.—Inhibition of respiration by 0.05M malonate, pH 4.1, and recovery following replacement of malonate by distilled water. Tissue used 44-216 hours after cutting.

These new experiments were done with roots from a batch of Danvers Half-long carrots dug in November and also with carrots bought on the open market. Similar results were obtained from all samples. The technique was as described already except that respiration was measured in M/15 KH_2PO_4 buffer at pH 4.5, with or without inhibitor and succinic acid. In all experiments the rate of oxygen uptake in buffer alone was measured before the acids were

tipped from side-arms; in experiments of type *A* (Fig. 10) the malonate was added first, the succinate (or water as control) later. In type *B* (Fig. 10)

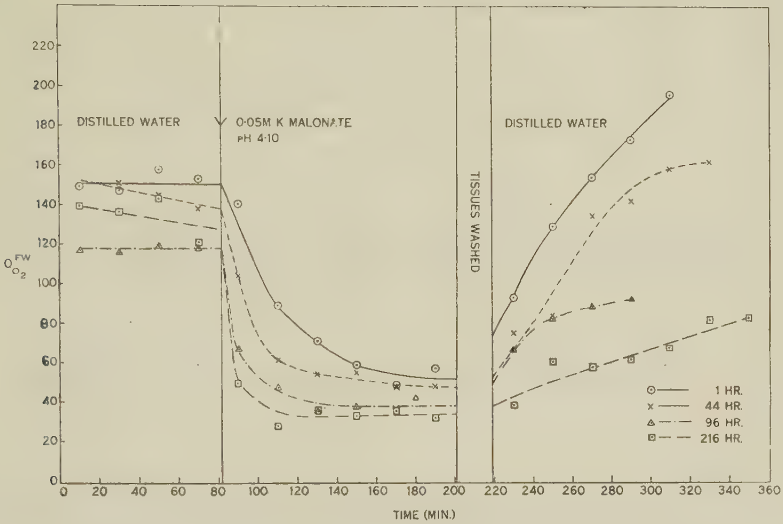

Fig. 7.—Inhibition of respiration by 0.02-0.03M malonate, pH 4.1, and recovery following replacement of malonate by distilled water. Tissue used 1-216 hours after cutting.

both acids were added together, and controls with single acids were run at the same time.

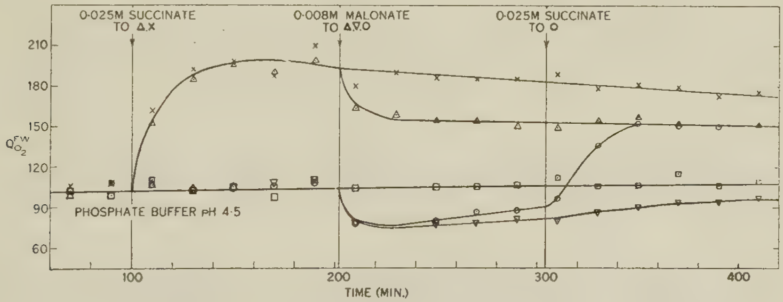

Fig. 8.—Experiment illustrating reversal of malonate inhibition of respiration by succinate and competitive inhibition. Five sets of carrot root slices, 310 hours washed, in M/15 phosphate buffer pH 4.5. Malonate at 0.008M, which does not bring the $Q^{FW}_{O_2}$ to the basal level even in the absence of succinate.

Examples of the results are plotted in Figures 8, 9, and 10, and a concise summary of all these experiments is given in Table 2 and Figure 10. The single figures for Q_{O_2} in this table represent the mean values during each period of treatment, excluding values for transition periods (e.g. the first 30 minutes after tipping acids). Most of these figures are means of 12 ten-minute readings.

TABLE 2

REVERSAL OR PREVENTION OF MALONATE INHIBITION BY SUCCINATE. ALL EXPERIMENTS WITH M/15 KH_2PO_4, pH 4.5

Type *A* experiments, malonate added first, succinate later (reversal)

			Malonate			Succinate plus Malonate			Succinate	
Expt.	Hours from Cutting	Initial Rate $Q^{FW}_{O_2}$	Malonate Concentration (M)	$Q^{FW}_{O_2}$	Percentage of Initial Rate	Succinate Concentration (M)	$Q^{FW}_{O_2}$	Percentage of Initial Rate	$Q^{FW}_{O_2}$	Percentage of Initial Rate
W 46/2	290	108	0.008	82	76	0.025	138	128		
3	310	106	0.008	85	80	0.025	150	142		
4	335	116	0.008	99	85	0.025	154	133		
W 52/1	93	160	0.05	37	23 Mean	0.025	36	23 Mean		
		173		39	23 **21**	0.01	34	20 **21**		
		160		28	18	0.008	30	19		
		136	0.01	84	62	0.025	178	131		
		152		96	63	0.01	206	136		
		143		101	71	0.008	211	148		
		160	0.008	80	50	0.025	201	126		
		155		78	50	0.01	171	111		
		178		104	75	0.008	191	107		
		147	—			0.025			244	166
W 52/2	117	135	0.03	33	24 Mean	0.025	41	30 Mean		
		148		36	24 **26**	0.008	44	30 **30**		
		159		44	28	0.001	46	29		
		145	0.01	88	61	0.025	220	152		
		127		71	56	0.008	174	137		
		140		87	62	0.001	174	124		
		149	0.005	109	73	0.025	210	141		
		141		94	67	0.008	202	143		
		155		111	72	0.001	192	124		
		148	—			0.025			230	156
W 79/1	140	177	0.05	112	63	0.025	188	106		
		173		110	64		185	107		

TABLE 2 (*Continued*)

Type *B* experiments, malonate and succinate added simultaneously (competition)

			Malonate			Succinate plus Malonate			Succinate	
Expt.	Hours from Cutting	Initial Rate $Q^{FW}_{O_2}$	Malonate Concentration (M)	$Q^{FW}_{O_2}$	Percentage of Initial Rate	Succinate Concentration (M)	$Q^{FW}_{O_2}$	Percentage of Initial Rate	$Q^{FW}_{O_2}$	Percentage of Initial Rate
W 46/2	290	99	0.008			0.025	160	162		
		110	0.008	82	76	—				
W 52/3	235	143	0.01			0.025	174	122		
		137	0.01	126	92	—				
W 53/1	65	144	0.005	106	74	—				
		167	0.01	93	56	—				
		170	0.01			0.025			228	137
		172	0.01			0.005			214	124
		167	0.01			0.001			167	100
		162	0.005			0.025	262	162		
		135	0.005			0.005	226	167		
		166	0.005			0.005	138	83		
		174	0.01			0.025	243	140		
		171	0.01			0.005	198	116		
		190	0.01			0.001	134	70		
W 58/1	90	110	0.01	59	54	—				
		109	—			0.025			241	221
		111	0.01			0.025	97	88		

It was not always practicable to run a control set of slices in buffer throughout, so all comparisons are based on initial readings in buffer (see Fig. 8).

These results make it clear that for carrot tissue, inhibition by malonate is reversible so long as concentrations of malonate are used that are not high enough to inhibit completely to the ground level. The inhibition is apparently of the competitive type, its extent being much reduced when malonate and succinate are added together—in fact, a mixture of the two acids usually brings the respiration rate above the initial steady value in buffer. Failure of reversal was obtained in two experiments, in both of which the malonate (0.03-0.05M) brought the respiration to the "ground" level (Figs. 9, 10). Reversal was always obtained when the respiration had been inhibited by malonate to a lesser extent, even for instance in Experiment W 79 (Table 2), when the malonate was at 0.05M and the succinate only 0.025M.

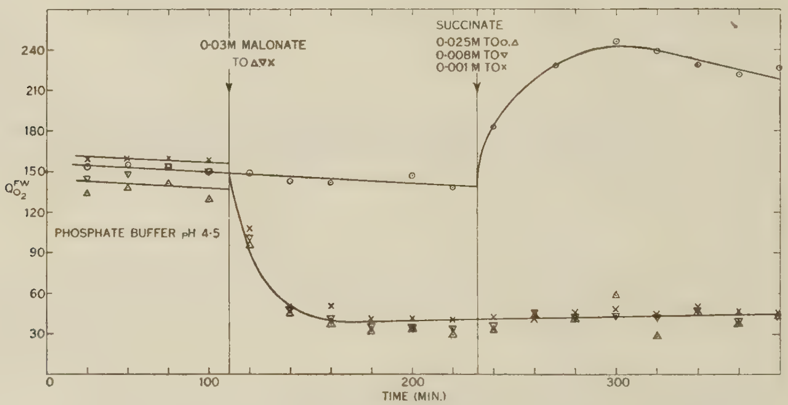

Fig. 9.—Experiment illustrating lack of reversal by succinate of malonate inhibition of respiration, when the malonate concentration (0.03M) has brought the respiration down to the ground level. Four sets of carrot root slices, 117 hours washed in M/15 phosphate buffer at pH 4.5.

In the experiments (type *A*) with weak malonate (0.005-0.01M) self reversal of inhibition had begun before the succinate was added, but nevertheless the marked effect of succinate was not thereby obscured.

Figure 10 shows the relationship between the concentration of succinate and the degree of reversal of malonate inhibition. It is, of course, possible that still higher concentration of succinate might cause reversal of the inhibition brought about by 0.05M malonate. As stated above, such reversal with the concentration ratios of 0.05M malonate/0.25M succinate was not obtained in earlier work. It is, however, difficult to test this point adequately because high acid concentration (near 0.2 or 0.3M) is harmful to the tissue. The simplest explanation of all our results (and of those of Machlis) is that malonate concentrations sufficiently high to bring the respiration down to the ground

level inhibit succinic dehydrogenase and also other enzyme systems concerned in the organic acid cycle; reversal with succinic is therefore not to be expected.

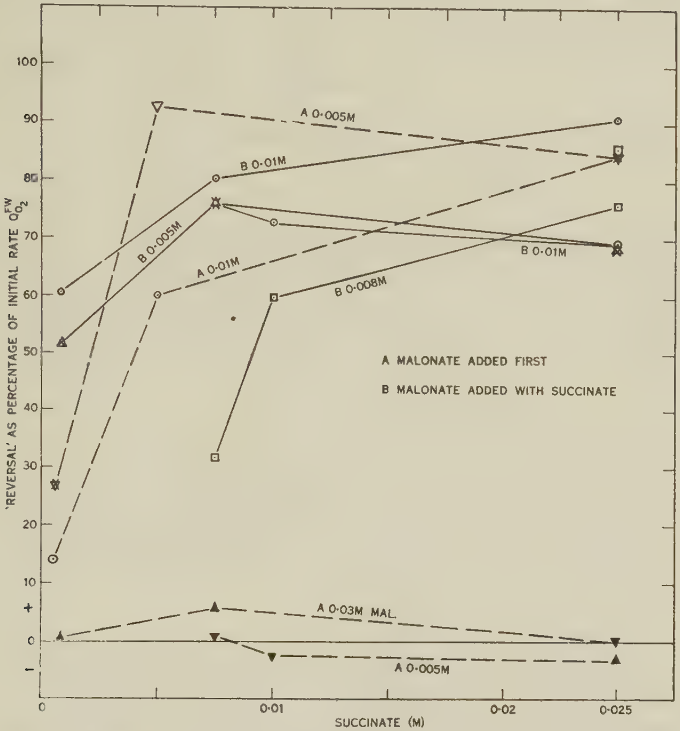

Fig. 10.—Effect of succinate in reversal of malonate inhibition. Succinate concentration plotted against the difference between $Q_{O_2}^{FW}$ malonate and Q_{C}^{FW} malonate + succinate, expressed as a percentage of the initial rate in phosphate buffer. *A*, malonate added first; *B*, malonate and succinate added together. The figures on the lines give the concentration of malonate.

It should be noted that the difference between the later experiments and the earlier was not due simply to the presence of a buffer solution; in the first place we obtain no reversal in buffer solution if the malonate has inhibited to the basal rate; secondly, we have confirmed reversal of malonate inhibition for carrot root slices unbuffered by phosphates, using 0.01M malonate and 0.025M succinate.

(v) *Effects on the Organic Acid Content of the Tissue* (*pH* 4-4.5)

Bonner (1948) and Laties (1949*b*) found that for oat coleoptiles, spinach leaves, and barley roots the normal low level of succinic acid in the tissues is raised following the addition of malonate at pH 4.5. The succinic acid accumulation is regarded as due to its oxidative formation from pyruvate and

4-C dicarboxylic acids, either those present in the cell or those formed by CO_2 fixation with pyruvate. In some spinach samples and in barley roots, added pyruvate and fumarate enhance the succinic accumulation associated with malonate.

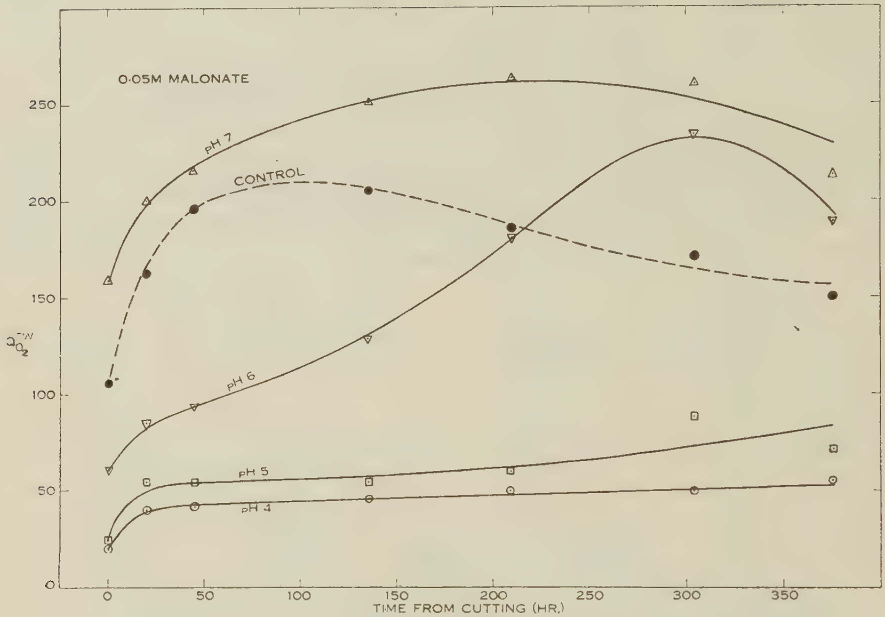

Fig. 11.—Drift of the respiratory response of carrot root tissue towards 0.05M malonate with time from cutting of the slices. The values plotted are the minimum (or maximum) rates of oxygen uptake achieved in the several experiments; control, tissue in distilled water. Each point gives the result of a four-hour experiment with tissue taken from the batch washing in water (see Figs. 12, 13).

Experiments along these lines have been carried out in this laboratory with carrot tissue, the organic acids being detected and, in some experiments, quantitatively estimated by the methods of Lugg and Overell (1948) and Bryant and Overell (1951).* The carrot root tissues, as used in our respiration experiments, invariably contained malic acid (whose concentration fell with age from cutting); in most samples smaller but measurable quantities of succinic acid were also present.

In six experiments we have only twice obtained any accumulation of succinate as a result of the addition of malonate (0.015-0.05M, pH 4.0); in neither case was the increased succinate content significant. In the other four

* The tissues were extracted with 80 per cent. boiling ethanol and the extract, after removal of alcohol, passed through an Amberlite I R A 400 column. Anions were eluted with 1N ammonium carbonate, and acids assayed by paper chromatography. We are indebted to Mr. Overell for advice and assistance in these experiments.

experiments there was no change in succinate concentration, although in two of them the malonate was added to the tissue respiring in an atmosphere of 4 per cent. carbon dioxide, in an attempt to promote carbon dioxide fixation. In all these six experiments the tissues were shown to contain malonic acid after treatment with this acid at pH 4 and the malonate supplied to replicate samples caused inhibition of respiration.

(c) *Effects of Malonate at pH* 5-7

(i) *Oxygen Uptake* (*pH* 5-7)

The results of a series of experiments, in which both the pH of malonate (0.05M) and the age of the slices were varied, are plotted in Figure 11. It will be clear that the effects of solutions more alkaline than pH 4.5 are different from those already described. Numerous experiments confirm those of Table 3 and the effects may be summarized as follows:

TABLE 3

RESPIRATION RATES FOR ONE TIME-SERIES OF EXPERIMENTS WITH 0.05M K MALONATE SOLUTIONS, pH 7-4

	Respiration rates (cu. mm. O_2/g. fresh wt./hr.)				
Time from Cutting (hr.)	Control in Water*	pH 7 Maximum Rate	pH 6 Minimum or Maximum Rates, i.e. Turning Point of Curve	pH 5 Minimum Rate—Turning Point of Curve	pH 4 Minimum Rate
1	106.2	159.0	60.0	24.0	21.0
20	165.0	201.0	84.0	54.0	40.0
44	197.4	216.0	93.0	54.0	42.0
136	206.4	252.0	129.0	54.0	47.0
208	185.0	264.0	180.0	60.0	50.0
304	172.2	261.0	234.0	87.0	50.0
376	142.8	213.0	189.0	72.0	56.0

* Mean of the steady rates of the four tissue sets in distilled water before malonate solutions were tipped.

Malonate 0.05*M, pH* 5 (0.5 *per cent. undissociated molecule* H_2M, 82 *per cent. monobasic ion* HM'.—This solution has an effect similar to that of weaker malonate at pH 4 (0.01-0.02M). It slowly depresses the oxygen uptake (but not to the basal level). There is always, however, a reversal of the inhibition with time, the rate rising during a four-hour period towards the control rate in water (Figs. 12, 13). The minimum rate reached is a function of the age of the tissue slices, tending to rise from $Q^{FW}_{O_2} = 50$ (or in a few experiments, 20) to 80 as the age increases from 1 to 400 hours, Table 3). The rate of recovery from the initial depression is greater for the older slices (Fig. 13). The pH of the external malonate solution shows little change during these experiments.

Laties (1949*a*) showed also that in barley roots the inhibition at pH 5 falls off with time.

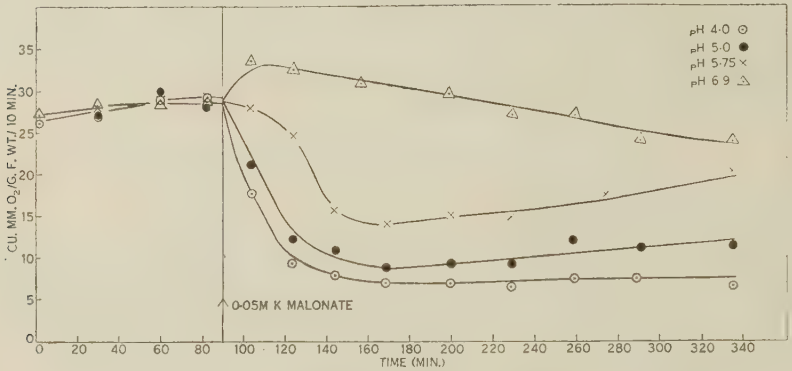

Fig. 12.—Reactions of tissue respiration towards potassium malonate solutions (0.05M) at four different pH. Tissue used 20 hours after cutting.

Malonate 0.05M, pH 6 (0.02 per cent. undissociated molecule H_2M, 32 per cent. monobasic ion HM′).—This solution has effects that vary markedly with the age of the slices (Figs. 11, 12, 13). Tissue that has been cut less than 200 hours reacts in the same way as tissue supplied with solutions at pH 5,

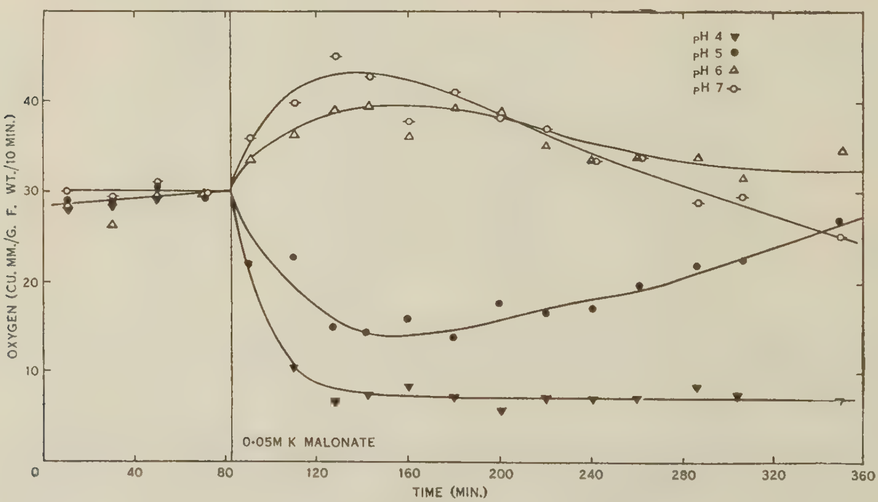

Fig. 13.—Reactions of tissue respiration towards potassium malonate solutions (0.05M) at four different pH. Tissue used 308 hours after cutting.

except that the rate of inhibition is less and the minimum value reached before recovery is higher. At 200 hours the solution at pH 6 has no apparent effect on the oxygen uptake, whereas after this time, stimulation is obtained as with neutral malonate (Figs. 3, 4). These solutions, at pH 6, did not show any marked drift of pH with time during the experiments.

Malonate 0.05*M*, *pH* 7 (*salt completely dissociated*).—This solution consistently causes an initial stimulation of the oxygen uptake (Figs. 11, 12, 13, 14); subsequently the rate returns either to that normal in water or to a value slightly below this. The stimulation is caused whatever the age of the tissue slices, although it is greater for the older tissues. During these experiments the pH of the external solutions falls to about 6.

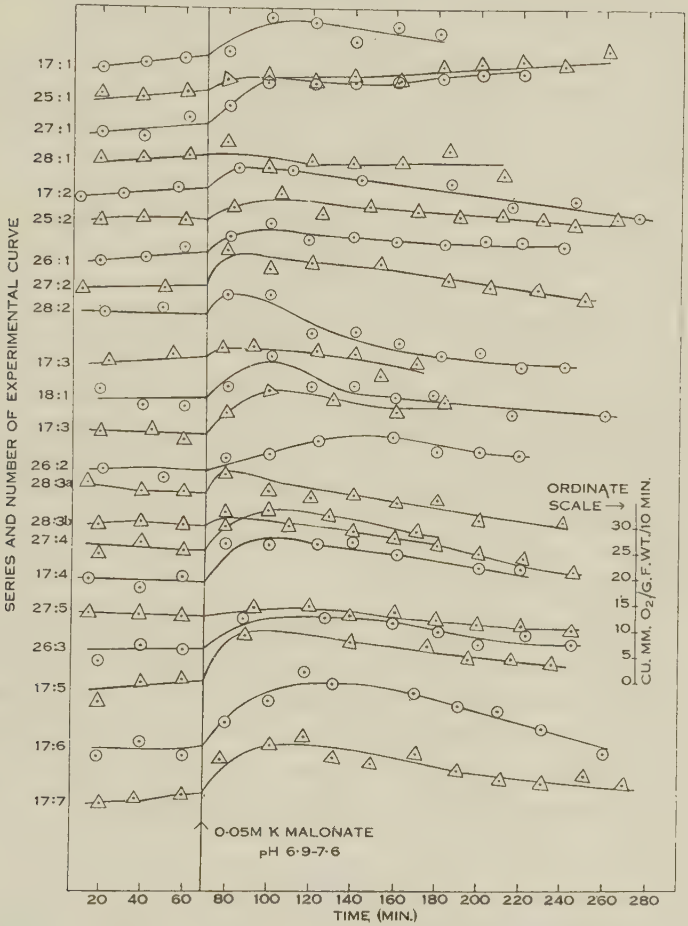

Fig. 14.—Reaction of tissue respiration towards solution of potassium malonate, 0.05M, pH 6.9-7.6. Periods of washing 2-308 hours.

(ii) *Respiratory Quotients; pH* 7

These were measured by the two-vessel method of Warburg. The reliability of the method for carrot tissue, when the pH is high, is discussed by Turner and Hanly (1949). In those experiments in which the pH of the malonate was 7.0 the two-vessel method is inaccurate owing to the presence of bicarbonate in the solution: the results were therefore corrected by using

several supplementary vessels in which the carbon dioxide retention was measured by acid tipping.

In contrast to the high R.Q. in malonate at pH 4-4.5, the R.Q. of tissue respiring in neutral potassium malonate solution is below unity during the early stages of the experiment. The extent of the "bicarbonate" error and the true change in R.Q. with time are illustrated in Table 4; accompanying the R.Q. change there is a fall in pH of the medium from 7 to about 6.

TABLE 4

RESPIRATORY QUOTIENTS IN NEUTRAL MALONATE SOLUTIONS

Malonate solution, initial pH 7.3, causing stimulation of $Q_{O_2}^{FW}$	
R.Q. over first 200 min. after tipping	0.68 Direct method
	0.76 Corrected by acid tip
R.Q. over second 200 min.	0.98 Corrected
R.Q. over whole period (400 min.)	0.75 Direct method
	0.85 Corrected
Final pH, in vessels with potash absorbent 6.2	

As far as can be ascertained in short manometric experiments, the corrected R.Q. following malonate addition at pH 7 is significantly below unity (0.8-0.9) for some one to two hours, after which it drifts upwards to a value of unity or even slightly above this. We have no R.Q. figures for malonate effects at pH 5 and 6.

(iii) *Reversibility; pH* 5-7

Only a few experiments were made to discover the effect of replacing the malonate solutions by water; the transfer was always made two hours after malonate addition. At pH 5 and 6 there was a rapid recovery of the $Q_{O_2}^{FW}$ from the depressed level (self reversal not being complete) to levels above that normal for water. At pH 7 the $Q_{O_2}^{FW}$ was increased slightly above the level reached previous to malonate stimulation.

(*d*) *Discussion*

(i) *Inhibition at pH* 4-4.5.—Malonic acid is clearly effective as an inhibitor of carrot root respiration only at the lowest pH experimentally tested, viz. 4-4.5. Solutions at this pH, of concentration 0.03-0.05M, usually bring the oxygen uptake down to a steady state (the "ground or basal respiration"). Lack of recognition of the importance of pH is almost certainly responsible for early statements that plant respiration is not inhibited by malonate.

The simplest hypothesis to explain the pH effect on inhibition is that the malonic acid inhibits the enzymic activity only when it is in the un-ionized form. Clear evidence of this might be provided by experiments of the kind described for azide by Stannard and Horecker (1948). Unfortunately at low

concentrations of malonate at pH 4 and higher concentrations at pH 5 and 6 the effect produced by malonate is complex (depression followed by recovery and concomitant change of R.Q.). The form of any curve plotting percentage inhibition with pH will vary with the total concentration, the age of the disc, and the time at which percentage inhibition is determined.

Stannard and Horecker (1948) found that azide was more effective as a respiratory inhibitor in frog muscle at low pH. They also obtained a similar pH effect with extracted enzyme systems and they showed that both with cyanide and azide the cytochrome-oxidase-inhibitor complex is formed only with the undissociated acids HN_3 and HCN. *In vitro,* succinic dehydrogenase from the carrot root is hardly active at pH 4 but at least the evidence suggests that since malonate is fully effective as the enzyme inhibitor at pH 7, it must act as an ion.

If, then, we assume (with Potter and Dubois 1943) that one of the malonate ions is the effective inhibitor, we still have to explain the marked pH effect on inhibition *in vivo.* Bonner published a single curve of inhibition *versus* pH for one concentration of malonate and concludes that because this "closely follows the titration curve of malonate the pH effect is due to the penetration only of HM′." Other workers on cellular inhibitors (e.g. Thimann and Schneider 1938; Simon and Blackman 1949) assume that the cell possesses an external membrane relatively impermeable to ions but permeable to molecules. The argument would then be that malonate enters the cell only as the molecule and dissociates within the cytoplasm to give the reactive ion. Although there is good evidence for such differential permeability for the tonoplast, there are ample reasons for doubting that the cytoplasm is much more permeable to uncharged than to charged particles. Moreover, the internal equilibrium concentration of malonate ions would not be determined by the ease of entry of molecules, but by the Donnan effect.

Robertson and Wilkins (Robertson 1951) have shown that the entry of anions from KCl is greater at low pH. They explain this as due to the suppression of ionization of weak electrolytes (which provide indiffusible ions in the cytoplasm) by H^+ ions from the medium. According to the Donnan effect, this would allow greater entry of anions from the medium. Such an explanation would of course apply also for malonate and with such a weak acid the pH effect would be enhanced because at pH 4 the external concentration of HM′ is maximal (Fig. 1). Various complicating factors may well be concerned, however, e.g.:

(i) Loss of inhibitor ions from cytoplasm to vacuole;

(ii) Exchange of K^+ in medium for H^+, tending to increase the internal pH;

(iii) Buffering of the internal pH by the system sugar $\rightleftharpoons$ acids as shown by Ulrich (1941);

(iv) Reversal of inhibition by malonate if acids of the Krebs cycle are produced in (iii).

With such a complex system, it seems impossible to interpret the pH effect on inhibition in any simple fashion.

The rapid recovery after washing is in accord with the view that malonate forms a dissociable complex with succinic dehydrogenase; the increased $Q_{O_2}^{FW}$ after washing away the malonate suggests that during the period of inhibition some intermediate substrate accumulates which can rapidly be metabolized only in the absence of malonate. The R.Q. data support this view. During the inhibition of respiration the R.Q. is *c.* 3 and fermentation is occurring. After washing, the R.Q. falls rapidly but it nevertheless remains above unity (*c.* 1.3) even after respiration has risen above the normal level. Such a high R.Q. and stimulation of respiration would be the accompaniment of organic acid metabolism; accumulation of succinate in the presence of malonate has been established for some tissues, but up to the present we have been unable to demonstrate it for carrot tissue.

(ii) *Stimulation at pH* 7.—In complete contrast to its effect at pH 4, malonate at pH 7 causes an initial stimulation of oxygen uptake by carrot root tissue. Unlike the similar stimulation brought about by KCl, this is accompanied by a low R.Q. of 0.8-0.9 and it is followed by a slow downward drift of the respiration to or below the control value in water, the R.Q. rising concurrently to unity or more. Burris and Wilson (1939) suggested that malonate may be respired at pH 7, but there is no direct evidence for this. We prefer to adopt the hypothesis that at pH 7 the internal concentration of malonic ions is kept low and that the stimulation of respiration is connected with the presence of K^+ in high concentration in the medium (Fig. 1). We presume a ready exchange of K^+ for H^+ and a tendency for the internal pH to rise (in our experiments the pH of the external solution consistently decreased to about 6, but this was no doubt in part due to carbon dioxide retention). According to Ulrich (1941) the internal pH rise due to cationic exchange is countered by the formation of organic acids (cf. according to Burstrom (1940) by malic acid). Such a process could well lead to high oxygen uptake and a low R.Q., while increased organic acid would also be expected to reduce malonate inhibition by competition.

The fall in pH of the external solution from 7 to 6 during these experiments provides us with a possible explanation of the fall in respiration rate which succeeds the initial rise, as at pH 6 malonate normally brings about partial inhibition.

An alternative explanation of the stimulation caused by malonate at pH 7 is that the salt acts in the same way as KCl, which stimulates respiration and is accumulated in the vacuole. A similar suggestion has already been made for succinate (Turner and Hanly 1949); it is not inconceivable that an ion might be transported across the cytoplasm by a carrier and thus be unable to play its part as an inhibitor. The concept of "salt stimulation" by malonate would, however, be more acceptable if it could be shown that malonate accumulates in the vacuole.

(iii) *Partial Inhibition, Followed by Recovery.*—Partial and temporary inhibition of the malonate-sensitive fraction of the respiration is induced either

by sub-optimal concentrations (0.01M) of malonate at pH 4.0 or by higher concentrations (0.05M) at pH 5 or 6 (Table 5). In such solutions the oxygen uptake is partially inhibited ($Q^{FW}_{O_2}$ 40-80); the inhibition is not maintained, but the rate rises until complete recovery to the normal rate is effected, usually within three hours. In Table 5, solution *a* causes maximal inhibition to the basal level, while *b* and *c* produce the partial temporary inhibition described above. One possibility, already discussed, is that solutions *b* and *c* act alike because both provide low concentrations of malonic molecule. It is more likely, however, that the tendency in *b* for the low pH to favour penetration of HM′ is counterbalanced by the low concentration of HM′, whereas in *c* the higher pH is associated with higher [HM′].

TABLE 5

IONIZATION AND MALONATE EFFECTS

	pH	Malonate Added	$[H_2M]$ $\times 10^{-4}$M	[HM′] $\times 10^{-3}$M	[M″] $\times 10^{-4}$M	Type of Inhibition
a	4.0	0.05M	30	46	10	Complete to basal level
b	4.0	0.01M	6	9	2	Partial and temporary
c	5.0	0.05M	3	40	87	

For solutions of the type here concerned, the rate and degree of initial inhibition by a given concentration increase as the pH is altered from 7 to 4. (This is so at least for discs of one particular age.) The characteristic feature, however, is what may be termed self recovery from inhibition. This has also been reported for sub-optimal strengths of malonate with other tissues; for the carrot it only occurs when the inhibition of the malonate-sensitive respiration is incomplete. After such reversal to the normal rate the respiration may be again inhibited to the ground rate by the addition of cyanide, and hence we presume that recovery is probably due to reversal of the enzyme inhibition and recovery of the system operated by cytochrome and succinic dehydrogenase, previously inhibited by malonate at the succinic dehydrogenase enzyme.

It has been shown that added succinate can, under certain circumstances, reverse malonate inhibition and the most likely explanation of self reversal is the formation of succinate in concentration sufficient to compete with malonate for the enzyme. We have not been able to demonstrate accumulation of succinic acid in tissues in which inhibition to the basal level is complete; but it is possible that when inhibition is incomplete and only succinic dehydrogenase is inhibited, accumulation of succinic acid by oxidative formation may be sufficient to reverse the malonate inhibition. This is the explanation for self reversal given by Laties (1949*a*). Reversal may well be accelerated if the "Ulrich" effect noted above is operating to increase the concentration of organic acids.

The malonate effects at pH 7 and 4 are fairly uniform for discs of very varied age from cutting. The effects at pH 6, however, vary markedly with the "age" of the discs, as is shown in Figure 11; also by comparing Figures 12 and 13. For example, at pH 6.0, malonate applied to freshly cut tissue produced a clear-cut but partial inhibition of the malonate-sensitive respiration; in tissue washed for 300 hours the malonate caused stimulation; while for tissue washed for only about 200 hours, the malonate had no effect on the value of oxygen uptake. These results are possibly connected with changes in the nature of the permeability and nutritional status of the cells during aging and they may eventually throw some light on the nature of the so-called wound respiration in tissue slices.

(iv) *The Krebs Cycle.*—Although proof of the existence of a Krebs cycle in carrot is lacking, the facts described above fit well with the view that malonate at low pH inhibits part of the respiration of carrot tissue by inhibiting the succinic dehydrogenase link in such a cycle. Succinic acid is metabolized by this tissue and succinic dehydrogenase has been obtained from it and shown to possess many of the characteristics of the animal enzyme — for instance, it is competitively inhibited by malonate. The inhibition *in vivo* may be removed by washing away the malonate and this is in accord with the view that malonate forms a dissociable complex with succinic dehydrogenase. Moreover, we present evidence for the reversal of the inhibition by addition of succinate. Our earlier failure to obtain reversal (cf. also Machlis 1944) was clearly due to the use of too high a concentration of malonate. In view of what is known about the effect of malonate on other enzymes of the Krebs cycle, it appears probable that, for carrot tissue at least, when concentrations greater than 0.04M are applied, the cycle is interrupted at more than one place (although the same fraction of the respiration is inhibited). Hence reversal by the addition of succinate alone would not be expected.

The evidence for the operation of a Krebs cycle in carrot tissue would be stronger if it could be shown that application of malonate at the pH that allows respiratory inhibition also brings about the accumulation of succinate, oxidatively, in the tissue. So far our experiments have failed to show such accumulation. We suggest that such accumulation does, in fact, begin when the actual inhibitor concentration in the cell is low, but that it cannot then be demonstrated because it leads to reversal of inhibition. In tissue in which inhibition of respiration is complete to the basal level, we take it as highly probable that the inhibitor concentration is such that not only S.D., but also other enzymes of the Krebs cycle are put out of action. Accumulation of succinate when the S.D. is inhibited is only possible given a continual supply of oxaloacetate and pyruvate. We have no reason to think that oxaloacetate could not be supplied in such tissue from such a source as aspartic acid, but if its oxidation is inhibited by high concentration of malonate, then succinate accumulation could not occur. Under such conditions one would expect the pyruvate from glycolysis to be diverted to give fermentation products and it is

significant that in the carrot tissue in which succinate accumulation is not demonstrable, the R.Q. accompanying malonate inhibition is very high (*c.* 3). On the other hand, for tissues in which succinate accumulation does occur, much lower R.Q.'s have been recorded; calculation shows that the R.Q. of succinate formation would be less than 1 if the necessary oxaloacetate came by carboxylation of pyruvate; 1.5 if it came from a pre-existent store in the cells; and again less than this if aspartic acid were the source.

(v) *Basal Respiration.*—In many plant tissues, respiration is not reduced to zero by cyanide or malonate; in carrot tissue the curve relating respiration to the concentration of either of these inhibitors approaches an asymptote at a rate of the order of $Q_{O_2}^{FW} = 30\text{-}40$, although it is true that with further increase in the inhibitor concentration, the curve takes a downward course again (Fig. 2). This latter effect may be ascribed to secondary inhibitions and the view is put forward that in carrot tissue, as in some other plants, there is a basal respiration mediated by enzyme systems resistant to cyanide, malonate, and carbon monoxide.

The autotrophic respiration of *Chlorella* is stated to be completely cyanide-resistant (Genevois 1927), and James and Beevers (1950) report that the respiration of *Arum* spadix tissue is not inhibited either by cyanide or by malonate at pH 3.5. Commoner (1940) has reviewed the evidence for a cyanide-resistant basal respiration in other tissues. More recently Warburg, using as evidence the hyperbolic nature of the curve "per cent. inhibitor/[CN]"," states categorically that inhibition by cyanide is as complete as is required by the dissociation law. But Warburg has ignored Commoner's argument concerning the danger of expressing inhibition in terms of percentage of the control, while, for the tissues he quotes, the basal respiration, if existent, may be of a very low order. As noted above, we find that the effects of malonate on carrot respiration are closely similar to those brought about by cyanide and carbon monoxide. The basal rates attained in all three inhibitors are of the same order; other evidence (Robertson and Turner 1945; Weeks and Robertson 1950) is available that supports the view that the cyanide- and CO-sensitive factor of the respiration is mediated by the cytochrome oxidase system, and that salt accumulation is not possible if this fraction of the respiration is inhibited. We take it that malonate, acting on the succinic dehydrogenase linked to the cytochrome oxidase system, also inhibits the cyanide-sensitive system and a *prima facie* case is made out for the existence of a basal respirator. Investigation of the enzyme systems (e.g. of flavoprotein type) concerned in such basal respiration is long overdue.

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THE INFLUENCE OF TEMPERATURE ON THE RATE OF DEVELOPMENT OF INSECTS, WITH SPECIAL REFERENCE TO THE EGGS OF *GRYLLULUS COMMODUS* WALKER

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Summary

Eggs of the cricket *Gryllulus commodus* Walker† (Gryllidae, Orthoptera) were incubated at nine constant temperatures; the mean duration of the incubation period and its variance have been determined at each temperature.

The mean rate of development of the eggs, expressed as percentage development per day, has been plotted against temperature, and a logistic curve having the form

$$Y = \frac{14.0906}{1 + e^{5.848176 - 0.223386t}},$$

derived from the data by the method of maximal likelihood, is shown drawn through the observed points. The observed points can be seen to lie closely along the calculated curve, yet the departures of the observed means from the calculated values, though small, are highly significant.

Two other cases in which logistic equations had been used to express the trend in rate of development of insect eggs with change in temperature and which on visual inspection appear to be excellent fits, have been re-examined and in each the observed means depart significantly from the calculated values.

The departure of the observed means from the theoretical curve is shown to increase as the period of development is extended to include post-embryonic stages of development.

Two or more developmental stages differing in their response to change in temperature may not be considered together when attempting to express trend in rate of development by means of a logistic curve. Since all stages during embryogenesis are unlikely to respond in exactly the same way to change in temperature, it is concluded that the logistic curve cannot be used to express precisely the trend in the rate of development of eggs at different temperatures. Whether the trend of a stage of development responding uniformly in its rate of development to change in temperature conforms to a logistic curve remains an open question.

I. Introduction

Many attempts have been made from time to time to describe the quantitative relationship that exists between temperature and the rate of development of poikilothermic animals by means of mathematical equations. The

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† *Gryllulus commodus* Walker has long been known in the Australian literature as *G. servillei* Saussure. The former name has been adopted on the authority of Dr. L. Chopard of the Paris Museum, to whom specimens were submitted through the Commonwealth Institute of Entomology, London. See also Uvarov (1935).

earlier literature on this subject has been reviewed by Belerádek (1935), Needham (1942), and Fry (1947).

In 1942, Davidson proposed the use of the logistic curve, developed by Pearl and Reed (1920) for use in describing the growth of populations, to express the relationship between temperature and the rate of development of insects and in 1944 he elaborated this concept and gave an outline of a graphical method of deriving the constants in the logistic equation. He concluded that the logistic, when so derived, followed the observed trend in rate of development more closely and over a wider range of temperatures than any other curve in use up to that time. Also in 1944, Birch applied the method of maximal likelihood in estimating the parameters in the logistic equation and found what was apparently a statistically insignificant departure of the observed mean values from the expected values calculated from the logistic equation.

The present paper is concerned with an analysis of results obtained for the eggs of the Australian black field cricket, *Gryllulus commodus* Walker, and a re-examination of some earlier data in the light of this analysis.

II. Experimental Material and Methods

A large number of crickets were caught as late instar nymphs in the field at Blackwood, near Adelaide, and were kept in humid cages in a glasshouse until they reached maturity. They were fed on grass and wheat grains. When most of the crickets had reached the adult stage, shallow trays of moist sand were introduced into the cages into which the females oviposited freely.

The trays were removed daily and the eggs sieved out under water. All the eggs laid on any one day were collected together, labelled, and placed in an incubator at 12.8°C., where they remained for 30-40 days. This cold temperature treatment was necessary since the eggs at the time of laying were either in a state of diapause or diapause supervened soon after, and it was known that with about 30 days treatment at 12.8°C., it was possible to obtain eggs uniformly free from diapause. During this period little or no morphological development occurred. Diapause will not be considered further here as it forms the subject of a later paper.

Experiments were done at nine constant temperatures (see Table 2 for the ranges of the thermostats). For each temperature treatment five batches of 50 eggs each were used, each batch being placed on moist blotting paper in a petri dish. The petri dishes were placed on stands in air-tight glass jars in the bottom of which was 100 ml. of distilled water, since previous experience had shown that unless the relative humidity in the jars was close to 100 per cent. most of the eggs died.

The number of nymphs that had hatched was recorded every 24 hours at temperatures up to and including 23.3°C. From 25.8 to 28.7°C. inclusive counts were made every 12 hours and at 31.5 and 33.8°C. every 8 hours. The shorter intervals were necessary at the higher temperatures to equalize, as far as possible, the loss of information for each temperature treatment.

Temperature readings were taken at 9 a.m. each morning and the mean value assessed from these.

Since it was not possible to do all nine temperature treatments concurrently a control experiment was done at 26.8°C. with each group of treatments. Thus the temperature treatments 16.4, 19.4, 21.1, 23.3, and 26.8°C. (control I) were done together, treatments 25.8, 28.7, and 26.8°C. (control II) together, and treatments 31.5, 33.8, and 26.8°C. (control III) together. The second control lot, II, contained only three batches or 150 eggs, but since it was observed for hatchings every 12 hours, whereas I and III were observed only every 24 hours, it was used to estimate the duration of the incubation period at this temperature.

TABLE 1

MEAN DURATION OF THE INCUBATION PERIODS OF THREE LOTS OF EGGS USED AS CONTROLS

Control No.	Mean Duration of Incubation Period (hr.)
I	309.6
II	314.9
III	314.3

ANALYSIS OF VARIANCE OF CONTROL LOTS

Source of Variation	Degrees of Freedom	S.S.	M.S.	V.R.
Between lots	2	3502	1751	7.66*
Error	584	133549	228.7	
Total	586	137051		

* Significant at $P = 0.001$.

Analysis of the differences in the mean duration of the incubation period in the three control lots of eggs is shown in Table 1. The difference in the mean of I from those of II and III, though small, is highly significant. This difference may be due to either or both of two causes; the eggs in II and III may have been different from those in I in developing more slowly, or the environment in which they developed may have been different. Control I was incubated in a different thermostat from II and III because at the time the latter were started this was needed for other work. Examination of the daily temperature records of the two thermostats used suggests that the mean temperature for the whole period was about 0.1°C. higher for I than for the other two. This is due to the difficulty involved in setting two thermostats to the same temperature. Examination of Figure 1 shows that a difference of this order would be sufficient to account for the difference in the mean duration of the incubation periods.

The analysis of variance presented in Table 1 shows that the precision obtained in estimating the residual variance has made it possible to demonstrate the significance of as small a difference as about one-quarter of the group interval used in estimating the means.

From these considerations, those treatments for which lot I was the control, namely 16.4, 19.4, 21.1, and 23.3°C., were included in the observations as "normal" eggs.

III. Results

Table 2 sets out the mean duration of the incubation period and the mean percentage development per day for eggs at each of the nine constant temperatures. The ranges of the thermostats are given in parentheses in column 2.

Table 2

OBSERVED MEAN DURATION OF THE INCUBATION PERIOD AND PERCENTAGE DEVELOPMENT PER DAY AT SEVEN CONSTANT TEMPERATURES

Array No.	Temperature (°C.)	Mean Duration of Incubation Period (days)	Mean Percentage Development per Day ($\bar{y}_i$)
1	19.4 (± 0.4)	39.59	2.5295
2	21.1 (± 0.5)	29.34	3.4121
3	23.3 (± 0.1)	20.26	4.9433
4	25.8 (± 0.1)	14.95	6.6979
5	26.8 (± 0.1)	13.12	7.6377
6	28.7 (± 0.2)	11.25	8.9107
7	31.5 (± 0.1)	9.28	10.7989

The mean percentage development per day at any temperature is estimated according to the equation

$$\bar{y}_i = \frac{\Sigma f\, 100/d}{n},$$

where

$\bar{y}_i$ = the mean percentage development per day,

f = the numbers of eggs that had hatched at each observation,

d = the duration of the incubation period at each observation, and

n = the total number of eggs hatching at the particular temperature.

Davidson (1944), following a graphical method of determining the constants in his equations as given by Pearl (1930), showed that when percentage development per day is plotted against temperature the resulting points appear to lie very closely along a logistic curve whose equation is

$$Y = \frac{K}{1 + e^{a + bt}},$$

where

Y = percentage development per day,

K = the parameter representing the interval between the upper and lower asymptotes of the curve,

a = the parameter determining the relative position of the origin of the curve on the abscissa,

b = the parameter determining the slope and course of the curve, and

t = temperature (°C.).

Subsequently Birch (1944) employed the method of maximal likelihood to determine the constants of the best-fitting logistic curve and the same procedure has been used here, giving the equation

$$Y = \frac{14.0906}{1 + e^{5.848176 - 0.223386t}},$$

for the data in Table 2. This curve is shown in Figure 1 plotted through the observed points.

The observed values of percentage development per day for the temperature arrays at 16.4 and 33.8°C. have been omitted in the calculation of the

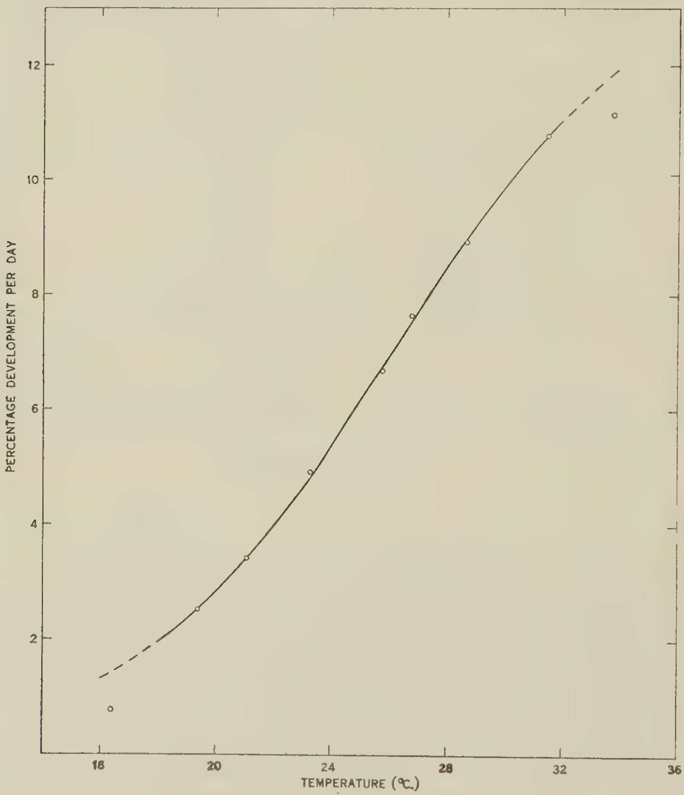

Fig. 1.—Logistic curve of form $Y = \frac{14.0906}{1 + e^{5.848176 - 0.223386t}}$ for the development of the eggs of *Gryllulus* drawn through the observed means.

curve since it is clear from Figure 1 that at both these temperatures development was retarded below the expected rate.

Visual inspection of Figure 1 indicates that all the observed points for temperatures between 19.4 and 31.5°C. inclusive lie closely along the calculated curve, but it is necessary to apply an objective statistical test to assess the goodness of fit.

Table 3 sets out the calculations involved in testing the goodness of fit of the calculated logistic. In column 5 are shown the deviations of the observed means from expectation $(\bar{y}_i - Y_i)$, whilst in column 6 the values of s_i^2, *the variance of the mean for each temperature array,* are given. The figures in column 2 are the numbers of observations on which each mean is based.

χ^2 is estimated according to the equation

$$\chi^2 \doteq \Sigma(\bar{y}_i - Y_i)^2/s_i^2,$$

where

$\bar{y}_i$ = the observed mean percentage development per day for any temperature array,

Y_i = the calculated value of percentage development per day for that array, and

s_i^2 = the estimated variance of the mean of the array.

χ^2 has $4 = (n - 3)$ degrees of freedom.

TABLE 3

GOODNESS OF FIT OF CALCULATED LOGISTIC CURVE FOR THE RATE OF DEVELOPMENT OF THE EGGS OF *GRYLLULUS COMMODUS*

Temp. (°C.)	n	$\bar{y}_i$	Y_i	$(\bar{y}_i\text{-}Y_i)$	s_i^2	χ^2
19.4	212	2.5295	2.5402	—0.0107	0.000034	3.37
21.1	227	3.4121	3.4281	—0.0160	0.000065	3.94
23.3	222	4.9433	4.8543	+0.0890	0.000162	48.90
25.8	232	6.6979	6.7468	—0.0489	0.000294	8.13
26.8	142	7.6377	7.5327	+0.1050	0.000900	12.25
28.7	231	8.9107	8.9778	—0.0671	0.000725	5.99
31.5	234	10.7989	10.8000	—0.0011	0.001181	0.00
Total				—0.0183		82.57

χ^2 at $P = 0.05$ with 4 degrees of freedom = 9.5.

The test must be performed in this manner since the intra-array variances differ significantly. It should be pointed out that the test is only approximate since the estimated array variances have been substituted for the unknown true variances, but in this particular case the use of the true χ^2 distribution will yield an accurate estimate of the probability since the array variances have been estimated with considerable precision.

When this test of goodness of fit was applied to the calculated curve, χ^2 was found to be significantly too large, 82.6 (Table 3, column 7), the value of χ^2 at $P = 0.05$ being 9.5.

A comparison between columns 5 and 6 in Table 3 shows that the differences between the observed and calculated values of percentage development per day are very large, relative to the standard deviations of their respective array means, at all temperatures except 31.5°C.; so large in fact that differences of this order over the seven arrays are unlikely to be attributable to chance.

This result was surprising in view of the fact that Birch (1944) in his work with *Calandra oryzae* found an insignificant value of χ^2. Figure 2 shows

the logistic curve derived by the method of maximal likelihood for the eggs of *C. oryzae* at moisture level I drawn through the observed points; the figure is redrawn from the data in Birch (1944). Table 4 shows the correct details of the calculation of χ^2 for Birch's data. The value of χ^2, 29.6, contrasts strongly with Birch's result of 0.1821, the discrepancy being due to the fact that Birch, in making his calculations, was following advice in which the intra-array variances were unfortunately substituted for the variances of the array means.

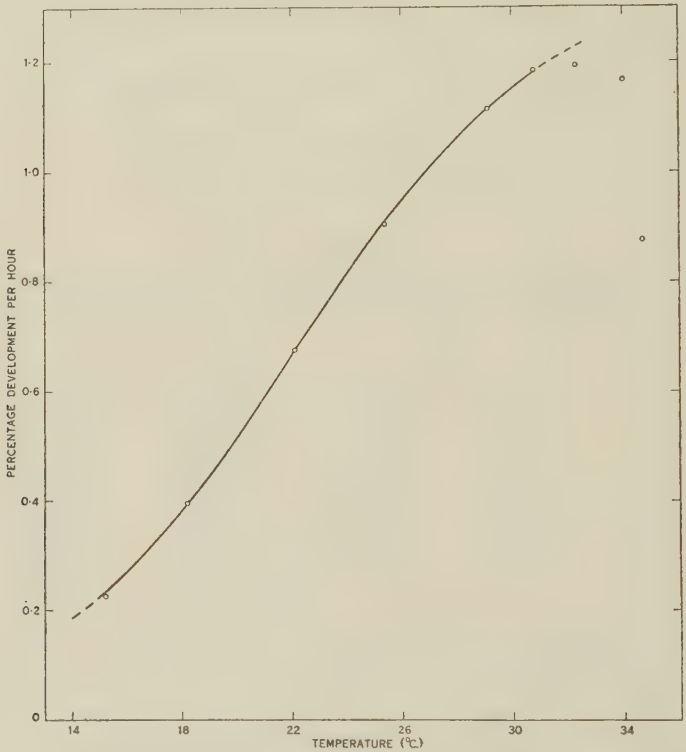

Fig. 2.—Logistic curve of form $Y = \dfrac{1.35062}{1 + e^{4.98145 - 0.2285t}}$ for the development of the eggs of *Calandra* drawn through the observed means (redrawn from Birch 1944).

It is evident that the observed values for percentage development per hour for these eggs also depart significantly from the calculated curve (at $P = 0.05$, $\chi^2 = 7.8$, with 3 degrees of freedom). Thus it is again found that the logistic curve is inadequate to express precisely the trend in rate of development of eggs with change in temperature, even though, in this case, elaborate precautions were taken to exclude as many variables as possible from the environment (cf. Birch 1944).

Further confirmation was sought by a re-examination of the data for *Drosophila melanogaster* given by Powsner (1935) and used by Davidson (1944) in proposing the use of the logistic curve. Figure 3 shows the logistic

derived by Davidson using the graphical method. It was not possible to use the iterative method of closer approximation on these data, as the only figures available were the mean durations of the incubation periods at the different constant temperatures and the standard deviations of these means. The frequencies and class intervals within each temperature array were, of course, not given in Powsner's paper.

In Table 5 are shown the values of $\bar{y}_i$ for each temperature array, arrived at by dividing 100 by the value for the mean duration of the incubation period at each temperature. This introduces a small error (cf. the method given on p. 99) which is, however, probably not sufficiently large to influence the calculations to any appreciable extent. The values $s^2\bar{y}_i$ given in column 5 were calculated from the standard deviation of each mean as given by Powsner, from the equation

$$s^2\bar{y}_i = \frac{100^2\, s^2\bar{x}_i}{\bar{x}_i^4}$$

where

$s^2\bar{y}_i$ = the variance of the mean percentage development per hour, and

$s^2\bar{x}_i$ = the variance of the mean duration of the incubation period, as given.

TABLE 4

GOODNESS OF FIT OF CALCULATED LOGISTIC CURVE FOR THE RATE OF DEVELOPMENT OF THE EGGS OF *CALANDRA ORYZAE* AT MOISTURE LEVEL I (DATA FROM BIRCH 1944)

Temp. (°C.)	n	$\bar{y}_i$	Y_i	$(\bar{y}_i - Y_i)$	s_i^2	χ^2
15.2	140	0.226	0.233	— 0.007	0.000002	22.4
18.2	215	0.395	0.394	+ 0.001	0.000003	0.4
22.1	197	0.677	0.671	+ 0.006	0.000007	5.1
25.4	186	0.905	0.911	— 0.006	0.000023	1.5
29.1	175	1.114	1.114	— 0.000	0.000044	0.0
30.8	192	1.184	1.181	+ 0.003	0.000061	0.1
Total				— 0.003		29.6

χ^2 at $P = 0.05$ with 3 degrees of freedom = 7.8.

Column 7 of Table 5 shows that the discrepancies between the observed means, $\bar{y}_i$, and the calculated values, Y_i, when considered over all temperature arrays are again too great to be attributable to chance. Strictly the χ^2 test used here may only be applied after the method of maximal likelihood has been used to determine the adjustable parameters in the logistic equation but it should give a sufficiently approximate estimate of the goodness of fit for the purpose at hand.

IV. Discussion

The application of the χ^2 test of goodness of fit to the three logistic curves presented in Figures 1, 2, and 3, shows that all three are inadequate representations of the observed trend in rate of development of eggs at different constant temperatures, despite the fact that on visual inspection the observed

points lie closely along the calculated curve. This is so even when the adjustable parameters of the curves have been derived by the method of maximal likelihood. Since this has been demonstrated for three independent sets of data, on the eggs of insects belonging to three widely separate orders, it can be expected to hold for all data of this kind obtained with corresponding precision.

Fig. 3.—Logistic curve of form $Y = \frac{7.095}{1 + e^{4.4514215 - 0.2071879t}}$ for the development of the eggs of *Drosophila* drawn through the observed means (redrawn from Davidson 1944).

Two explanations of this state of affairs are open; either the data are too crude to fit the true logistic trend, or the logistic curve does not express the trend in rate of development with changing temperature, as it has been assessed in the experiments.

With regard to the first of these two possibilities, examination of the columns of variances of the means given in Tables 3, 4, and 5 shows that the means have been determined with precision, owing largely to the high numbers of replicates used in the experiments. However, it may be that the assessment of the actual temperatures at which the eggs developed was not sufficiently precise. The temperatures used in the calculations are estimates of

the temperatures within the glass jars or vials used as cages in the experiments and these containers, because of their bulk, are much less subject to temperature fluctuations than are the thermometers used. In the data for *Calandra* the temperature of each experiment was calculated as the arithmetic mean of a large number of readings and so will be a precise estimate of the actual temperature at which the eggs developed. Powsner would seem to have

TABLE 5

GOODNESS OF FIT OF CALCULATED LOGISTIC CURVE FOR THE RATE OF DEVELOPMENT OF THE EGGS OF *DROSOPHILA MELANOGASTER* (DATA FROM POWSNER 1935)

Temp. (°C.)	n	$\bar{y}_i$	Y_i	$(\bar{y}_i - Y_i)$	s_i^2	χ^2
14.95	54	1.4728	1.4561	+ 0.0167	5.1×10^{-5}	5.4
16.16	182	1.7361	1.7666	— 0.0305	1.3×10^{-5}	71.1
16.19	153	1.7762	1.7758	+ 0.0004	1.4×10^{-5}	0.0
17.15	129	2.0534	2.0535	— 0.0001	2.6×10^{-5}	0.0
18.20	64	2.4155	2.3849	+ 0.0306	8.7×10^{-5}	10.7
19.08	94	2.6309	2.6819	— 0.0510	1.7×10^{-5}	156.0
20.07	82	2.9904	3.0313	— 0.0409	5.1×10^{-5}	32.7
22.14	57	3.7679	3.7880	— 0.0201	13.9×10^{-5}	2.9
23.27	135	4.1186	4.1963	— 0.0777	4.2×10^{-5}	145.3
24.09	188	4.4524	4.4825	— 0.0301	3.3×10^{-5}	27.4
24.81	217	4.7304	4.7235	+ 0.0069	1.4×10^{-5}	3.3
24.84	141	4.7506	4.7334	+ 0.0172	3.7×10^{-5}	8.0
25.06	37	4.9044	4.8046	+ 0.0998	23.7×10^{-5}	42.0
25.06	84	4.8996	4.8046	+ 0.0950	7.9×10^{-5}	114.4
25.80	196	5.1440	5.0357	+ 0.1083	4.7×10^{-5}	247.8
26.92	104	5.3333	5.3578	— 0.0245	6.8×10^{-5}	8.8
27.68	148	5.6306	5.5560	+ 0.0746	16.9×10^{-5}	32.9
28.89	83	5.7604	5.8368	— 0.0764	20.4×10^{-5}	28.7
28.96	95	5.8038	5.8517	— 0.0479	10.8×10^{-5}	21.0
29.00	232	5.8343	5.8602	— 0.0259	6.1×10^{-5}	10.9
30.05	148	5.9630	6.0667	— 0.1037	13.0×10^{-5}	83.0
Total				— 0.0793		1052.6

χ^2 at $P = 0.05$ with 18 degrees of freedom = 28.9.

assumed that the mid point between the extremes of temperature fluctuation was equivalent to the mean temperature. If the temperature in the thermostats did not rise and fall at the same rate this may introduce an error that would be considerable with a wide range of temperature fluctuation. But in fact Powsner was able to reduce the extreme fluctuations to very small dimensions (between ± 0.1 and ± 0.05°C. in the thermostats). Furthermore, he measured his temperatures to the nearest 0.01°C. on standardized thermometers. He also found that the short-term fluctuations in the thermostats were not reflected in any temperature change that he could measure within the vials. In the data for *Gryllulus* the mean was again assumed to lie at the mid point between temperature extremes but the fluctuations were a little greater in some instances than they were in either of the other two sets of data. and the

error introduced by this method would be correspondingly greater. However, since the same kinds of thermostats were used in all this work, the displacement of the mean from the mid point would be in the same direction at all temperatures and this would tend to minimize any influence this error might have, since its influence is merely to move the curve along the temperature axis but not to alter its shape. Provided the displacement is equivalent at all temperatures this error has no influence on any conclusions regarding the goodness of fit of the curve. In the data for *Gryllulus* this is not so (Table 2) but the introduced errors are likely to be small and in any case the results corroborate those obtained from the other two sets of data.

Table 4 shows that in the data for *Calandra* the lowest temperature array, 15.2°C., made by far the greatest contribution to the value of χ^2. This was also true when the data for moisture level II, presented by Birch (1944), were similarly analysed. This circumstance gave rise to some suspicion regarding the validity of including the 15.2°C. array in the analysis and it was decided to recalculate these data omitting this array. When this was done, again using the maximal likelihood solutions for the parameters in the logistic equation, it was found that χ^2 became insignificant ($P > 0.90$), indicating that the trend in the rate of development over this temperature range was expressed adequately by means of a logistic curve. A similar situation occurred with the data for moisture level II.

Now the 15.2°C. arrays were discarded from these analyses solely because they contributed the greatest proportion of the total value of χ^2, and this is not a sufficient reason for their exclusion (Yates 1933). Nevertheless, Birch's Table 3 shows that the mortality at this temperature was considerably higher than at the other temperatures used in the calculation of the curves, and this may indicate that this temperature was causing interference with development or hatching and should therefore be excluded. This was not stated by Birch in his paper, and indeed he included these arrays in his analyses, but in a letter he informs me that he considers it likely.

In the data for the other two species there is no indication that the extreme temperatures used lay outside the limits of temperature at which development proceeded without interference, yet the calculated curves depart significantly from the observed points. Both these curves were calculated from a greater number of observed points than the data for *Calandra* and it is quite likely, since a logistic curve can be calculated to fit three points exactly, that the fit obtained for *Calandra* when the array at 15.2°C. was discarded, was due, to some extent, to the reduction in the number of points to which the curve was required to conform.

The question then remains to some extent an open one but it would seem that the weight of evidence indicates that the data are sufficiently precise to enable any statistically significant departure of observed points from a hypothetical curve to be regarded as real and to make it unlikely that the observed trend in rate of development with temperature can be expressed precisely by means of a logistic curve.

Powsner (1935), as well as measuring the time required by *Drosophila* to complete embryonic development and hatch at a series of constant temperatures, also measured the mean time taken to reach the pupal stage from egg-laying and the mean time required to complete the pupal period, each at a series of constant temperatures. From these data logistic curves have been calculated by the graphical method that can be drawn through the observed points for percentage development per hour for the periods "egg-to-pupa" and "egg-to-adult." (The mean "egg-to-adult" periods were obtained by adding the corrected mean duration of the "egg-to-pupa" period to the mean duration of the pupal period at the same temperatures. The variances of these means were obtained by adding the variances of the two periods. This procedure is justifiable since the duration of the two periods was not correlated.)

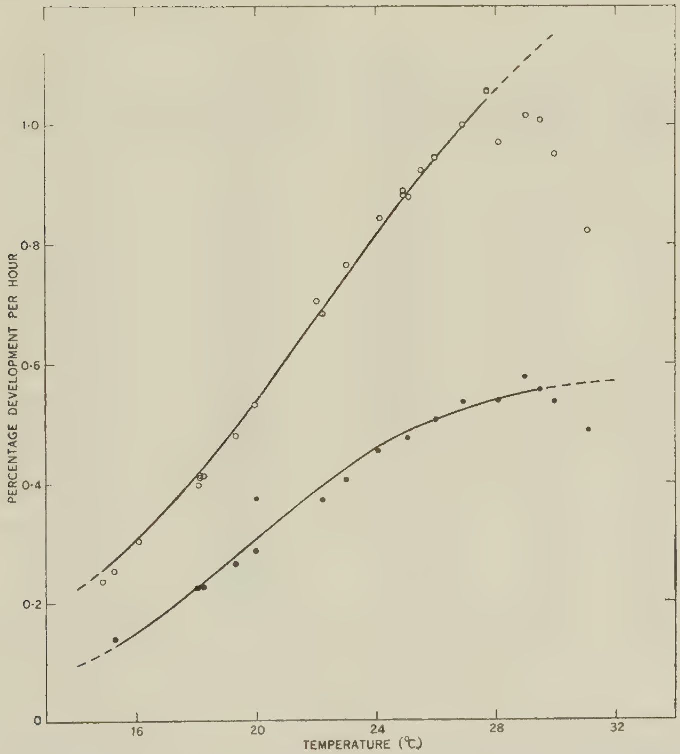

Fig. 4.—Logistic curves for the development of *Drosophila* drawn through the observed means. Upper curve, period of development from egg laying to pupation having the form $Y = \dfrac{1.376}{1 + e^{4.501606 - 0.203248t}}$; lower curve, period of development from egg laying to adult emergence having the form

$$Y = \frac{0.587}{1 + e^{5.748103 - 0.292319t}}.$$

Figure 4 shows these two curves drawn through the observed points, whilst Tables 6 and 7 show the calculations necessary to test the goodness of

fit of the two curves respectively. It can be seen on comparing these figures and tables with Figure 3 and Table 5 that the calculated logistic fits the data less and less well as the period of development under consideration is extended. Furthermore, the three periods of development show marked differences in their response to temperature; the optimum temperature for the egg stage being in the vicinity of 30°C., that for the egg-to-pupa stage about 27°C., and that for the egg-to-adult stage about 29°C. The points of inflexion of the three curves also differ markedly. Figure 5 shows the three curves drawn to the same scale and these differences can be clearly seen.

TABLE 6

GOODNESS OF FIT OF CALCULATED LOGISTIC CURVE FOR RATE OF DEVELOPMENT FROM EGG TO PUPA OF *DROSOPHILA MELANOGASTER* (DATA FROM POWSNER 1935)

Temp. (°C.)	n	$\bar{y}_i$	Y_i	$(\bar{y}_i - Y_i)$	s_i^2	χ^2
14.86	97	0.2365	0.2550	— 0.0185	88 × 10^{-8}	388
15.24	227	0.2527	0.2714	— 0.0187	32 × 10^{-8}	1097
16.06	148	0.3034	0.3095	— 0.0061	70 × 10^{-8}	54
18.04	99	0.3987	0.4165	— 0.0178	124 × 10^{-8}	255
18.05	423	0.4134	0.4171	— 0.0037	30 × 10^{-8}	47
18.05	178	0.4100	0.4171	— 0.0071	79 × 10^{-8}	63
18.21	81	0.4119	0.4266	— 0.0147	153 × 10^{-8}	142
19.32	122	0.4803	0.4957	— 0.0154	69 × 10^{-8}	343
19.97	125	0.5311	0.5383	— 0.0072	401 × 10^{-8}	13
22.00	128	0.7052	0.6779	+ 0.0273	80 × 10^{-8}	935
22.00	195	0.7057	0.6779	+ 0.0279	109 × 10^{-8}	712
22.21	139	0.6840	0.6918	— 0.0078	210 × 10^{-8}	28
22.99	220	0.7657	0.7470	+ 0.0187	124 × 10^{-8}	283
24.17	140	0.8439	0.8269	+ 0.0170	427 × 10^{-8}	67
24.93	242	0.8826	0.8778	+ 0.0048	119 × 10^{-8}	19
24.93	341	0.8913	0.8778	+ 0.0135	107 × 10^{-8}	170
25.14	1004	0.8795	0.8913	— 0.0118	135 × 10^{-8}	102
25.56	357	0.9234	0.9177	+ 0.0056	142 × 10^{-8}	23
25.99	344	0.9479	0.9441	+ 0.0038	158 × 10^{-8}	9
26.89	185	1.0000	0.9964	+ 0.0036	225 × 10^{-8}	6
27.77	128	1.0860	1.0436	+ 0.0124	244 × 10^{-8}	63
27.77	192	1.0604	1.0436	+ 0.0169	214 × 10^{-8}	133
Total				+ 0.0227		4952

χ^2 at $P = 0.05$ with 19 degrees of freedom = 30.1.

The fit of the three curves shown in Figures 3 and 4 may also be regarded as becoming progressively better as the period of development considered becomes shorter and presumably less heterogeneous in nature. Thus in the data for the egg-to-adult period three distinct developmental stages, each differing to some extent in their response to temperature, have been added together and the composite trend with temperature was found to depart significantly from a logistic curve. Now even if the trend of each of the three stages could be expressed precisely by a logistic, it would only be possible to compound them and fit the result to a logistic if the paramters a and b in the three

separate logistic equations were identical. In this particular case this is not so (cf. Davidson 1944 for the equations for the development of the eggs and of the pupae of *Drosophila*). The curve for the development of the larval stage differs even more widely. Thus it is not justifiable to consider the total developmental period as a unit when attempting to express the trend in rate of development with temperature in the form of a logistic curve. This being so it seems probable that the several parts of embryogenesis will behave differently, one from another, in their response to temperature. Such differences have been shown for stages in the embryonic development of the frog *Rana pipiens* (Ryan 1941). This is further borne out by work on lethal or sublethal temperatures with *Drosophila* eggs, in which it was shown that the influence of temperature depended on the stage of development of the embryo (Powsner 1935, Table XVI).

Table 7

GOODNESS OF FIT OF CALCULATED LOGISTIC CURVE FOR RATE OF DEVELOPMENT FROM EGG TO ADULT OF *DROSOPHILA MELANOGASTER* (DATA FROM POWSNER 1935)

Temp. (°C.)	$\bar{y}_i$	Y_i	$(\bar{y}_i - Y_i)$	s_i^2	χ^2
15.24	0.1398	0.1267	+ 0.0132	4×10^{-8}	4350
18.05	0.2239	0.2255	— 0.0148	3×10^{-8}	7300
18.21	0.2266	0.2320	— 0.0055	16×10^{-8}	188
19.32	0.2643	0.2787	— 0.0144	8×10^{-8}	2588
19.97	0.2873	0.3066	— 0.0193	38×10^{-8}	982
20.00	0.3729	0.3079	+ 0.0650	10×10^{-8}	42300
22.21	0.3702	0.3972	— 0.0270	20×10^{-8}	3630
22.99	0.4063	0.4259	— 0.0196	12×10^{-8}	3192
24.17	0.4527	0.4629	— 0.0102	40×10^{-8}	263
25.14	0.4757	0.4884	— 0.0127	13×10^{-8}	1238
25.99	0.5057	0.5071	— 0.0014	9×10^{-8}	22
26.89	0.5338	0.5236	+ 0.0102	23×10^{-8}	452
28.07	0.5371	0.5406	— 0.0036	118×10^{-8}	11
28.99	0.5577	0.5509	+ 0.0068	72×10^{-8}	65
Total			— 0.0333		66581

χ^2 at $P = 0.05$ with 11 degrees of freedom = 19.7.

It may be that if a small part of embryogenesis could be studied (say the period from fertilization to the completion of the blastoderm) which could reasonably be supposed to show a uniform response to temperature throughout, then perhaps it would be possible to demonstrate that the trend in rate of development with change in temperature (using even more rigorous methods of assessing the actual temperatures), could be expressed precisely by means of a logistic curve. Atlas (1935) measured the change in rate of development with changing temperature of the period from fertilization to the first cleavage division of the eggs of *Rana pipiens* but since he presented his data in the form of a figure only they are quite unsuitable for use in testing the validity of the logistic curve (cf. also Ryan 1941).

As yet, however, there is no hypothetical nor philosophical reason for supposing that the trend in rate of development at different temperatures should conform to the logistic or to any other particular curve. Much more must be known about the physiology of development before a hypothesis that can then be tested empirically can be formulated on this question.

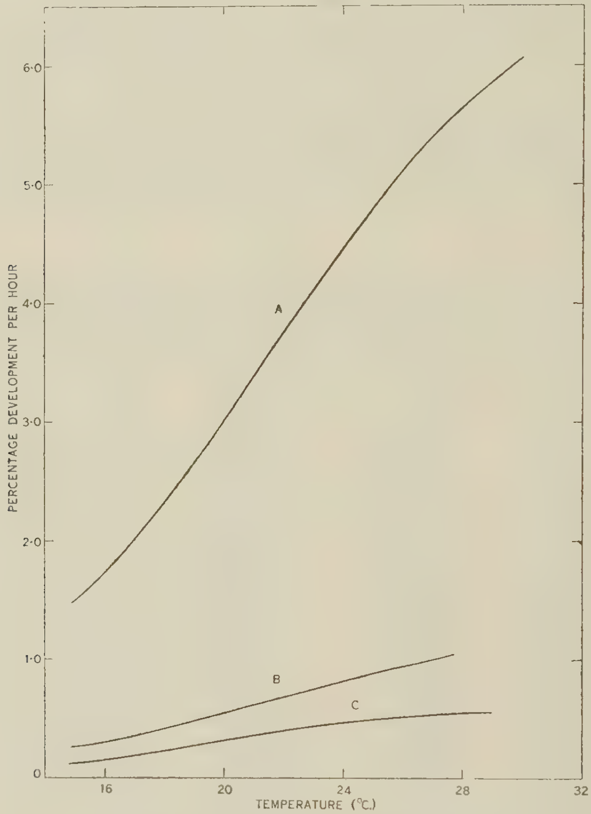

Fig. 5.—The three curves for the development of *Drosophila* drawn to the same scale.

A, development of eggs from laying to hatching.

B, development from egg laying to pupation.

C, development from egg laying to adult emergence.

From a physiological point of view then it remains an open question whether or not uniform stages of development, if such stages can be found, conform to the logistic in their trend in rate of development with temperature. It is certain, however, that if two or more such stages are considered together, the trend in their total development, supposing the several stages themselves show a logistic trend, cannot conform to a logistic curve unless they all respond in an identical manner to change in temperature (a and b in all the logistic equations must be identical).

From an ecological point of view, however, and it was as an ecologist that Davidson first proposed the use of the logistic, the logistic curve remains the most faithful representation of trend in rate of development of insects under changing temperature conditions. It can form a useful tool for predicting the rate of development at any particular temperature and the slight inaccuracies so introduced can be expected to be negligible when compared with the errors introduced by the estimations of field temperature unavoidable in ecological work.

V. Acknowledgments

It is a pleasure to acknowledge the kindness of Dr. E. A. Cornish, Section of Mathematical Statistics, C.S.I.R.O., in the generous way in which he gave so much time to this work. Dr. L. C. Birch, Department of Zoology, University of Sydney, kindly lent me some of his original data and helped with advice. I also wish to thank Mr. D. C. Swan, head of the Department of Entomology, Waite Agricultural Research Institute, for the interest he has shown in this work, and Dr. H. G. Andrewartha, also of this department, for his valuable advice and criticism given throughout the course of the work. Miss M. E. Morphett assisted with the technical work and prepared the figures.

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THE INFLUENCE OF TEMPERATURE ON THE COMPLETION OF DIAPAUSE IN THE EGGS OF *GRYLLULUS COMMODUS* WALKER

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Summary

The eggs of *Gryllulus commodus,* if incubated at high temperature (26.8°C.), soon after laying, did not develop and hatch promptly. Instead hatching was spread over a long period and many eggs died. If the eggs were given a period of exposure to low temperature (12.8°C.) before incubation at high temperature, prompt hatching occurred. This was due to diapause, which occurred at an early stage in the morphological development of the egg.

An experiment was done to measure the influence of various periods of exposure to high temperature followed by various periods of exposure to low temperature on the ability of the eggs to complete their diapause development.

It was found that a maximum period of about two days of high temperature treatment, followed by a minimum period of about 30 days low temperature treatment was most influential in promoting the completion of diapause.

About 20 per cent. of the eggs were found to be competent to develop without diapause when laid.

Preliminary high temperature treatment is considered to have induced the eggs to enter diapause more firmly than if no high temperature had been experienced before the exposure to low temperature. At the same time, high temperature permitted the completion of diapause, although under these conditions diapause development proceeded slowly and uncertainly.

It is shown that the processes responsible for hatching and melanin formation in the nymphs operate at low temperature but at a very slow rate.

I. Introduction

Diapause is a common phenomenon in the eggs of insects but great variability is found in its manifestation as between different species and even on occasions in the same species under varying circumstances. Among the eggs of any species there is always a distribution in the degree of intensity of diapause. For example, in species in which diapause disappears during an exposure to cold the intensity of diapause may be measured by the time required to be spent at low temperature. This may be very variable indeed and may be subject to influence by the environment, as will be shown later.

In some species all eggs enter diapause whilst in others the eggs are variable, some diapausing and some being competent to complete their development without interruption. In the latter type of species the proportions of diapausing and non-diapausing eggs are usually also very variable and dependent

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on many factors, including the physiological state and genotype of the mother, her environment, and the environments of the eggs themselves.

In most species that have been studied it is found that an exposure to low temperature, if given at the appropriate stage in the egg's development, results in the egg becoming competent to complete its development promptly when placed at some appropriate incubation temperature. However, it is found that the range of temperatures to which the diapausing egg may respond is usually extensive and may overlap the range of temperatures at which morphogenesis proceeds. Diapause usually disappears most rapidly at some moderately low temperature and more slowly at temperatures much above or below the optimum. At higher temperatures particularly, abnormalities and death frequently result from long exposures.

These considerations have led to the abandoning of the idea of diapause as consisting of a developmental "block" that had to be "broken" before development could proceed and given rise to the concept of diapause as a physiological process, part of the normal development of the individual, that must be completed before the morphogenetic processes can proceed smoothly. The recent work on hormones in relation to diapause leaves little doubt that the processes concerned in the completion of diapause are those of organization and development. These processes have been termed "diapause development" (Andrewartha 1952) and it is in this sense that the term is used here. Andrewartha has recently elaborated the concepts set out above in an extensive review of diapause in the ecology of insects and has cited detailed examples and references.

The present paper deals with the results of an experiment on the influence of varying periods of exposure to low temperature following varying periods of exposure to high temperature on the ability of the eggs of *Gryllulus commodus* to complete their development and hatch when placed at an adequate incubation temperature.

II. Materials and Methods

(*a*) *The Eggs*

About 50 female crickets caught in the field at Penola, South Australia, were kept in a large cage with about an equal number of males, with grass and wheat grains for food. A tray of moist sand was kept in the cage and examined daily for eggs, and when it became apparent that eggs were being laid freely the crickets were transferred in their cage to a warmed (25-30°C.) glass-sided box in a glass-house. Trays of moist sand were placed in the cage and these were removed at daily intervals and new ones replaced.

The sand was sieved under water and the eggs removed, cleaned, and sorted, any discoloured, undersized, or damaged eggs were discarded, and 1200 eggs were counted out. This procedure was repeated on each of three consecutive days since it was not possible to obtain all the 3600 eggs required for the experiment (see Section II (*b*)) on one day.

Each lot of 1200 eggs was immediately counted out into lots of 25, and two such lots, together representing one replicate, were allotted at random to each

treatment. Replicates were split in this way since the containers (2-oz. pomade jars with a quarter-inch of plaster of Paris set in the bottom) were too small to accommodate 50 eggs easily. The plaster was thoroughly wetted and the jars placed in the appropriate incubators. This procedure was repeated each day for three days, giving three replicates of 50 eggs each for each treatment.

(*b*) *Experimental Procedure*

Eggs of *G. commodus* that have completed diapause hatch in about 13.1 days at 26.8°C., whilst at 12.8°C. no eggs had hatched even after exposure to this temperature for almost a year (Browning 1951). Further, it was known that diapause development was successfully completed after about a month at 12.8°C. Consequently 26.8°C. was chosen as the high temperature, and 12.8°C. as the low temperature to be used in the experiment.

The eggs were given a preliminary exposure of 0, 2, 6, or 12 days to 26.8°C. and this was followed by a period of 0, 5, 15, 30, 45, or 60 days of low temperature treatment, after which the eggs were returned to the thermostat at 26.8°C. for incubation. The 24 possible combinations of these treatments were used, with three replicates of 50 eggs each of each treatment, giving a total of 3600 eggs in all. After the preliminary treatment the eggs were incubated for 60 days, after which time the experiment was stopped.

The jars in the incubation thermostat were opened daily, and hatched nymphs were counted and removed. Each week the plaster in the jars was moistened with a few drops of water in order to keep the humidity within the jar as close to saturation as possible, since previous experience had shown this to be necessary for the survival of the eggs.

The treatments will be referred to by number hereafter for the sake of brevity. Thus 0-15 means no high temperature treatment, followed by 15 days of low temperature treatment, 12-45, 12 days of high temperature treatment followed by 45 days of low temperature, etc.

Since the temperature at which the eggs were incubated after treatment was the same as that used in the preliminary high temperature treatment, the four treatments 0-0, 2-0, 6-0, and 12-0 are all similar since none had any time at low temperature between exposures to high temperature. They are thus the controls and in effect went straight to incubation with no preliminary treatment.

It should be stated here that treatment 6-15, through an oversight, was left too long at low temperature. All the replicates were moved at once as soon as the error was discovered, after 16, 17, and 18 days respectively, and they thus had one, two, and three days too much low temperature treatment. However, this is not a great difference and would certainly be negligible in its effect (no significant difference could be detected between the replicates) and for this reason the results of this treatment have been included as if it had in fact been 6-15.

III. Results

The distributions of hatching of the eggs in each treatment during the 60-day period are shown in Figure 1 in the form of cumulative hatching totals plotted against time. The ordinates in the figures represent the sum of the three replicates in each treatment, the maximum possible being 150. The points are joined by straight lines with a line parallel to the abscissa drawn from the last recorded hatching to the 60-day ordinate. The height of this line on the ordinate represents the total number of eggs that hatched.

It can be seen from Figure 1 that the hatching distributions in the various treatments differ in three main ways, namely: (*a*) in the total number of eggs that hatched during the 60-day period of observation; (*b*) in the mean duration of the incubation period of eggs that hatched, and (*c*) in the variance of the distributions. These differences will be considered separately.

(*a*) *Total Number of Eggs that Hatched*

The mean total number of eggs that hatched in each treatment, expressed as a percentage of the total eggs used, is set out in Table 1, in angular scale (Fisher and Yates 1948) with the actual mean percentages shown in parentheses. Such transformation of the data was necessary before the ordinary methods of statistical analysis could be applied (Bartlett 1947). Least differences for significance in comparing means are set out below the table.

Table 1

MEAN PERCENTAGE OF EGGS THAT HATCHED, IN ANGULAR SCALE

H.T. = 26.8°C., L.T. = 12.8°C.

L.T. Treatment (days)	H.T. (days) 0	2	6	12	L.T. Treatment General Means
0	39.6° (40.7%)	42.7° (46.0%)	39.2° (40.0%)	44.6° (49.3%)	41.5° (44.0%)
5	56.4° (69.3%)	58.6° (72.7%)	45.8° (51.3%)	53.6° (64.0%)	53.6° (64.4%)
15	75.0° (92.7%)	63.5° (79.3%)	48.5° (56.0%)	63.0° (79.3%)	62.5° (76.8%)
30	74.3° (92.7%)	71.6° (90.0%)	62.1° (77.3%)	74.1° (92.0%)	70.5° (88.0%)
45	74.4° (92.7%)	78.7° (96.0%)	67.8° (85.3%)	59.9° (74.7%)	70.2° (87.2%)
60	76.1° (93.3%)	74.6° (92.7%)	70.6° (88.7%)	60.1° (74.7%)	70.4° (88.8%)
H.T. treatment general means	66.0° (80.2%)	64.9° (79.4%)	55.7° (66.4%)	59.2° (72.3%)	

Least differences for significance at $P = 0.01$ between: individual treatment means = 11.7°; high temperature treatment general means = 4.8°; low temperature treatment general means = 5.8°.

Table 1 shows that in treatments receiving no or two days preliminary high temperature treatment the total number of eggs that hatched was about the same at any particular level of low temperature treatment. An increase in the total hatch occurred in both treatments as the period of low temperature treatment was extended to about 15 or 30 days, further increase having

no significant influence on the total. The number of hatchings was reduced, however, when the preliminary exposure to 26.8°C. was increased to six days,

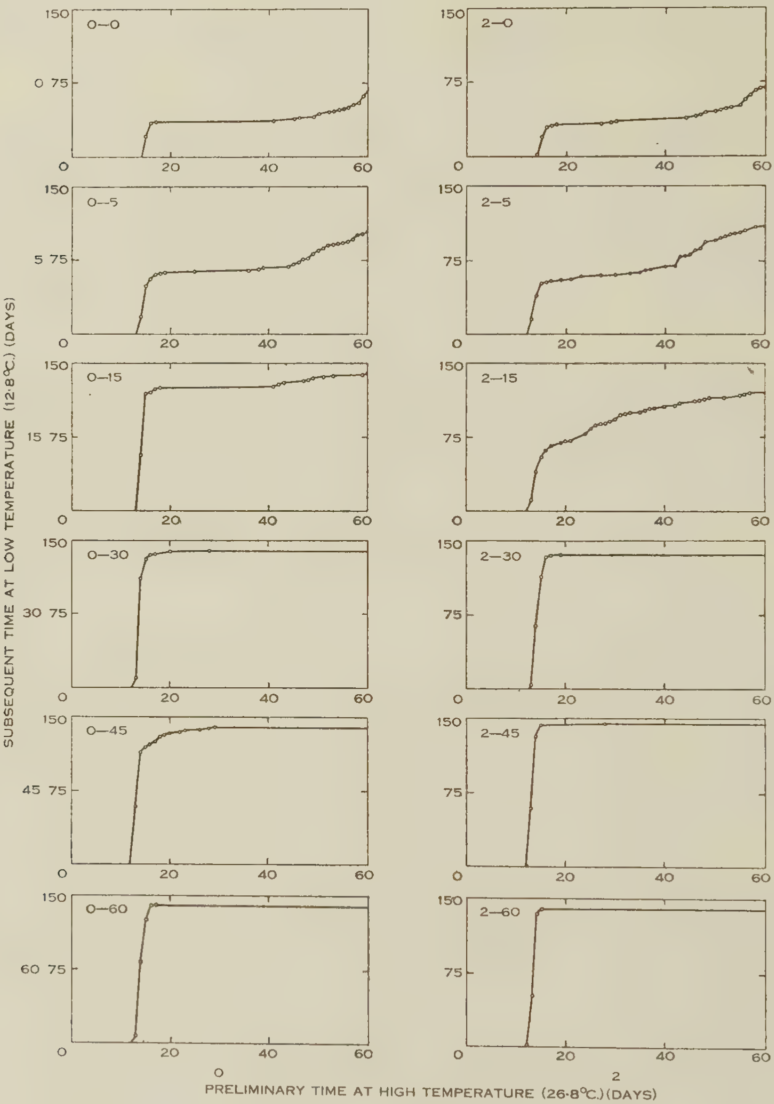

Fig. 1.—Cumulative frequency diagrams of hatching for all treatments, obtained by adding the results of all replicates of each treatment. In individual treatment graphs the ordinate represents the number of eggs hatched and the abscissa time in days.

but further increase to 12 days did not further decrease the number that hatched. In both the six-day and 12-day periods of preliminary high temperature treatment the number hatching increased as the subsequent period of

exposure to low temperature increased up to 30 days, but no significant increase occurred when this period was further extended.

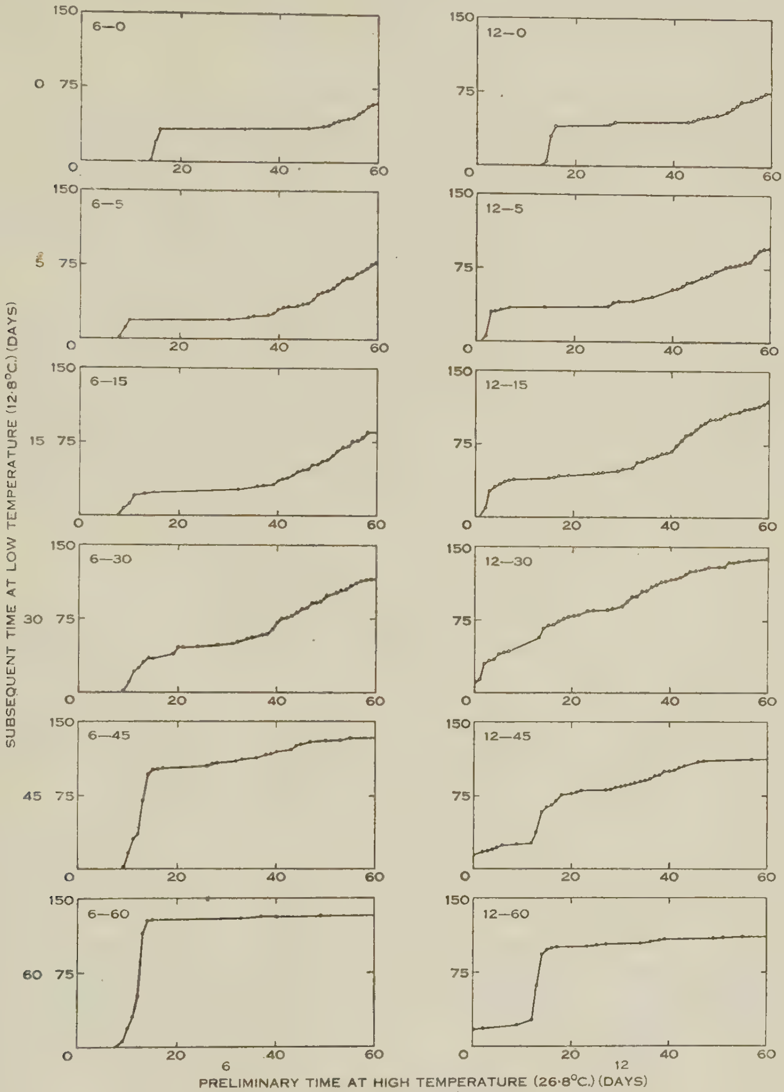

Fig. 1.—For explanation see opposite page.

In general, the preliminary period of treatment at high temperature had no influence on the total number of eggs that hatched unless it was extended beyond two days, whilst increase in the period of subsequent exposure to low temperature up to about 30 days resulted in an increase in the number hatching in all treatments. Further increase in the period of low temperature treatment had no significant influence. In general the number of eggs that hatched after experiencing a preliminary exposure to high temperature depended on

the duration of their subsequent exposure to low temperature; and the influence of low temperature in increasing the numbers that hatched depended on the duration of the preliminary exposure to high temperature. This is shown by a very highly significant variance ratio for the interaction between the high and low temperature treatments.

If the hatching of an egg is taken as the criterion that the embryo had successfully completed its diapause (or that diapause did not occur) then it is clear that extended periods of treatment at low temperature (30 days or more) were most influential in promoting diapause completion but that extension of the period of preliminary high temperature treatment greatly reduced the influence of the subsequent low temperature treatment.

(*b*) *Mean Duration of the Incubation Period*

In Table 2 are set out the means of the mean duration of the incubation period of those eggs that hatched following each treatment. It can be seen that increase in the duration of the preliminary high temperature treatment from nil to six days resulted in an increase in the duration of the incubation period, whilst further increase in high temperature treatment to 12 days was followed by a fall in the duration of the incubation period.

TABLE 2

MEAN DURATION OF INCUBATION PERIOD, IN DAYS

H.T. = 26.8°C., L.T. = 12.8°C.

L.T. Treatment (days)	H.T. (days) 0	2	6	12	L.T. Treatment General Means
0	33.5	35.0	33.4	30.8	33.2
5	28.3	28.5	39.2	30.5	31.6
15	17.6	22.2	40.7	29.0	27.4
30	13.8	14.2	32.0	20.3	20.5
45	14.0	13.2	18.2	18.7	16.3
60	14.0	13.2	12.6	13.4	13.3
H.T. treatment general means	20.2	21.5	29.3	23.8	

Least differences for significance at $P = 0.01$ between: individual treatment means = 7.6 days; high temperature treatment general means = 3.1 days; low temperature treatment general means = 3.8 days.

The duration of the incubation period became progressively shorter as the time spent at low temperature was increased from nil to 30 days in all cases except those receiving six days preliminary exposure to high temperature. Further increase in the duration of exposure to low temperature had no significant influence on the duration of the incubation period in treatments receiving nil or two days high temperature treatment whereas in treatments receiving six and 12 days high temperature treatment, increase in the duration of exposure to 12.8°C. up to 60 days continued to result in a decrease in the duration of the incubation period.

The very low values obtained for treatments 6-45, 6-60, 12-45, and 12-60 (Table 2) are due, in part, to the fact that a few eggs hatched very early after their return to high temperature for incubation (Fig. 1). These eggs must have completed a considerable part of their development during the long preliminary period of exposure to high temperature and then continued development, albeit very slowly, during the long periods spent at low temperature and so were almost ready to hatch on return to high temperature for incubation. A very small number of eggs in treatments 12-30, 12-45, and 12-60 were found to have hatched whilst at 12.8°C. This further assisted in reducing the mean duration of the incubation period following these treatments. Such eggs must have been virtually without diapause.

Eggs in which diapause had been successfully completed during the exposure to low temperature hatched promptly (after about 13 days) on return to high temperature for incubation and it can be seen from Figure 1 and Table 2 that the most influential treatments in reducing the average period required for incubation and so of promoting the successful completion of diapause were those in which nil or two days preliminary high temperature and 30 or more days low temperature treatment were given.

TABLE 3

MEAN LOG VARIANCE OF HATCHING DISTRIBUTION FOR EACH TREATMENT

H.T. = 26.8°C., L.T. = 12.8°C.

L.T. Treatment (days)	H.T. (days)				L.T. Treatment General Means
	0	2	6	12	
0	2.91 (406)	2.53 (343)	2.59 (388)	2.54 (350)	2.57 (372)
5	2.50 (319)	2.43 (272)	2.50 (334)	2.66 (465)	2.52 (348)
15	2.00 (108)	2.09 (130)	2.41 (275)	2.57 (376)	2.27 (222)
30	0.05 (2)	—0.21 (1)	2.45 (280)	2.43 (277)	1.18 (140)
45	0.77 (7)	—0.02 (2)	2.16 (147)	2.23 (186)	1.28 (85)
60	—0.68 (0)	—0.52 (0)	1.26 (20)	1.97 (95)	0.51 (29)
H.T. treatment general means	1.21 (140)	1.05 (125)	2.23 (257)	2.30 (292)	

Least differences for significance at $P = 0.01$ between: individual treatment means = 0.525; high temperature treatment general means = 0.208; low temperature treatment general means = 0.263.

(*c*) *Variance of the Distribution of Hatchings*

Figure 1 shows that in some treatments all the hatchings occurred during a few days and that in these cases the mean duration of the incubation period was also short, whilst in other cases hatchings occurred during a much longer period and often the mean duration of the incubation period was longer (cf. Table 2). Even in cases where the mean duration of the incubation period was short there were sometimes wide deviations from the mean among the individual hatchings. The variance of the hatching distribution during the 60 days of incubation was used as a measure of the variation in hatching time among the eggs in each treatment.

The variances were first transformed into logarithms and the analysis was performed on these. In Table 3 are set out the log variances, with the true variances in days^2 in parentheses.

It can be seen from Table 3 that in all treatments, except those receiving nil or two days preliminary high temperature treatment and 30 or more days subsequent low temperature treatment, the variances were very large, whilst in the latter treatments the variances were quite small. There was, however, a general tendency for the variances to be reduced as the period of low temperature treatment was extended.

Treatments in which most eggs hatched during a short period were those in which the diapause of the eggs had been successfully completed so that most eggs were ready to recommence their morphological development and hatch promptly on return to high temperature.

TABLE 4

DISTRIBUTION OF HATCHING OVER THE 60 DAYS OF INCUBATION* — *A*, WITH REGARD TO HIGH TEMPERATURE TREATMENT; *B*, WITH REGARD TO LOW TEMPERATURE TREATMENT

	Period (days)	0-7	8-11	12-20	21-40	41-60
A	H.T. Treatment					
	0	0	0	634	12	81
	2	0	0	575	52	88
	6	0	104	246	63	190
	12	156	5	215	115	160
	Total	156	109	1670	242	519
B	L.T. Treatment					
	0	0	0	139	8	122
	5	34	18	119	47	168
	15	37	6	219	66	133
	30	43	21	334	64	66
	45	24	31	401	46	26
	60	18	33	458	11	4
	Total	156	109	1670	242	519

* No attempt was made to analyse these results as they were very unwieldy from a statistical point of view and the differences seemed so great as to be self-evident. Also the replicates within each treatment agreed closely.

(*d*) *Distribution of Hatching*

Hatching was distributed very unevenly over the 60 days of observation when all eggs hatching during the experiment are considered. This is shown by the curves in Figure 1 but is demonstrated more explicitly in Table 4. In the table the 60-day observation period has been divided arbitrarily into five periods and the total number of eggs hatching during each period is shown with regard, in *A*, to the preliminary high temperature treatment and, in *B*, to the subsequent low temperature treatment.

All hatchings that occurred during the first seven days resulted from eggs that received 12 days high temperature treatment, and almost all the hatchings occurring during the following four days resulted from eggs that received six days high temperature treatment (Table 4*A*). In both these cases the subsequent low temperature treatment had very little influence on the number of eggs that hatched. These eggs must have completed a considerable part of their development during the treatment period and so must have been virtually without diapause (Table 4*B*).

The period from 12 to 20 days contained about three-fifths of the total number of eggs that hatched during the experiment and of these about three-quarters were due to treatments receiving nil or two days high temperature followed by 30 or more days low temperature. This period contains the modal period of incubation at 26.8°C. for eggs free from diapause (Browning 1952). Most of the hatchings occurring during the period from 21 to 40 days were due to treatments 12-15 and 12-30, the remainder being spread fairly evenly over all other treatments.

The final period was characterized by a marked rise in the total hatchings compared with the previous period, in fact it contributed about half the hatchings not occurring in the period from 12 to 20 days. Most of the total of 519 was contributed by the high temperature treatments 6 and 12 days which received 0, 5, or 15 days subsequent low temperature treatment. This was most probably due to the completion of diapause and subsequent development of these eggs even under conditions of continuous high temperature incubation. Diapause development required a much longer period and was much less certain to lead to the healthy hatching of the young nymph at high than at low temperature but nevertheless was successful in some cases.

(*e*) *Non-Diapause Eggs*

Figure 1 shows that in treatments in which no time was spent at low temperature (graphs 0-0, 2-0, 6-0, and 12-0), about 20 per cent. of the eggs hatched without obvious delay. The modal period of incubation of these eggs was between 14 and 15 days (see Table 4*B*, entries for no low temperature treatment). After this time few or no hatchings occurred until after about 45 days had elapsed. A comparison was made between the mean duration of the incubation period of all the eggs that hatched within the first 20 days in these four treatments (the treatments are in fact all similar: see Section II(*b*)) and the mean duration of the incubation period of all eggs that hatched following treatments 0-30, 0-45, and 0-60. The results are shown as a frequency distribution diagram in Figure 2. In Figure 2*A* the hatching distribution of all eggs from treatments 0-30, 0-45, and 0-60 have been divided by 3 to make them more easily comparable with those of Figure 2*B* which shows the distribution of hatching of all eggs from treatments 0-0, 2-0, 6-0, and 12-0 together. It is clear from the figure that hatching in eggs that hatched during the first 20 days of the experiment in treatments that received no low temperature treatment was distributed in a very similar manner to that in the treatments in which diapause had been completed at low temperature. The means

of the two distributions (13.9 days in Figure 2*A*; 14.8 days in Figure 2*B* shown by the broken lines), although significantly different, were very similar and lend weight to the conclusion that the early-hatching group of eggs in the control treatments developed virtually without diapause.

This conclusion is further substantiated by the fact that a group of eggs in treatments receiving 12 days preliminary high temperature treatment and some subsequent low temperature treatment hatched much sooner after return to incubation than any other eggs in the experiment (Table 4*A*). These eggs must also have been virtually without diapause since they were able to complete a substantial part of their development during their initial exposure to low temperature.

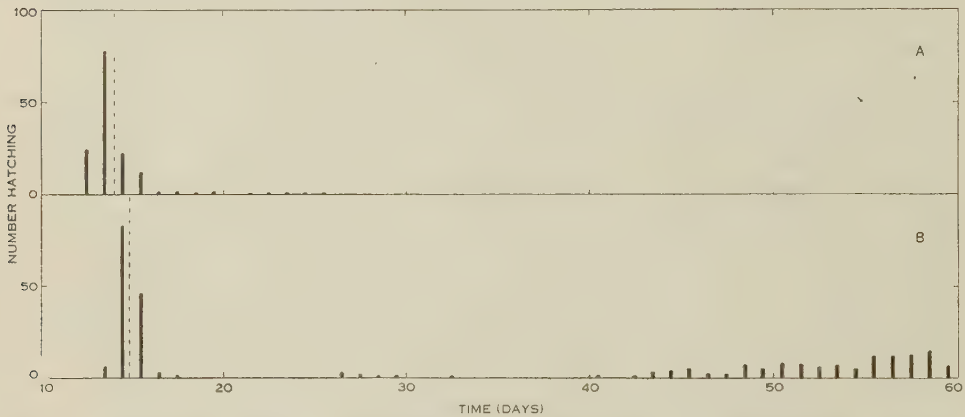

Fig. 2.—Frequency diagrams of the distribution of hatching of: *A*, all eggs from treatments with no preliminary high temperature treatment and 30 or more days low temperature treatment prior to incubation at 26.8°C. *B*, all eggs receiving no low temperature treatment and incubated from day of laying at 26.8°C. continuously for 60 days.

(*f*) *Pigmentation of Eggs and Hatching at Low Temperatures*

During the course of the experiment it was observed that certain eggs in treatments 12-30, 12-45, and 12-60, when removed from the low temperature cabinet, were quite black. It was also found that certain eggs in the above three treatments hatched whilst still at low temperature. Neither of these phenomena was observed in any other treatment.

Further consideration of these two observations will be deferred to Section IV.

IV. Discussion

In the experiment hatching was taken as the criterion of the successful completion of diapause. It is realized that this was perhaps an inadequate yardstick but it was considered to be the only one practicable in this case. Better criteria would have been the ability of young nymphs to feed and grow,*

* Dr. H. G. Andrewartha informs me that he was not able to induce nymphs of *Austroicetes cruciata* to feed when the embryonic diapause had been completed under experimental conditions, whereas nymphs hatched naturally in the field fed readily in captivity.

the ability of the eggs to produce fecund adults, or measurements could have been made of the oxygen consumption rate of the eggs and the time of completion of the diapause judged from this (Bodine 1932). None of these methods was practicable, however, and diapause was considered to have been completed if the egg hatched.

In a few instances nymphs were found to have broken out of the egg membranes but to have died before completing the first moult. Nevertheless, they were considered as having hatched. It seems from this that diapause may be completed under some circumstances but that development is in some way "unhealthy" or "abnormal." This is a subject that merits much more consideration than it has been given.

The experiment was stopped when each replicate, after treatment, had spent 60 days in the incubation cabinet. This also is a defect in the method but it was considered that the experiment could achieve its object even if eggs that would have hatched later than this were discarded.

It is usual to assume that the distribution of hatching of healthy eggs developing without diapause, when incubated at a particular adequate temperture, will be nearly normal about a particular mean and with a particular variance and that a high proportion of the eggs will hatch. It is known that in the eggs of *G. commodus*, in which the diapause was completed at low temperature, the mean duration of the incubation period at 26.8°C. is 13.1 days with a variance of 0.35 $days^2$ and that an average of 94 per cent. hatched (Browning 1952). Treatments producing results approaching these values then can be considered as having been influential in promoting the completion of diapause. For this reason, the three criteria chosen in the analyses of the results of the experiment were (1) total number of eggs that hatched during the 60-day period of observation, (2) mean duration of the incubation period, and (3) variance of the hatching distribution. A large value for the first and small values for the second and third indicate that diapause was at a minimum and that morphogenetic development was proceeding with a minimum of interruption.

It is evident from Figure 1 and Tables 1, 2, and 3 that the treatments in which the greatest numbers of eggs hatched in the shortest time and with the smallest "scatter" were those that received nil or two days of preliminary high temperature treatment and 30 or more days low temperature treatment prior to incubation. There was no increase in effectiveness as the low temperature treatment period was extended beyond 30 days nor was there much to choose between nil and two days preliminary high temperature treatment. Treatment 0-30 then exerted the maximum influence in the minimum treatment time.

Hatching in all treatments other than the six mentioned above showed some degree of departure from optimum conditions and so can be considered to have been less effective than these in promoting the completion of diapause in the eggs.

Figure 1 shows that with the exception of the above six treatments hatchings in all others occurred over a wide range in time. If hatching really means that diapause had been successfully completed, or did not occur, then this

"spread" of hatching must mean that there was a great variability in the intensity of diapause among the eggs. The progressive increase in the number of eggs that hatched promptly after return to incubation as the duration of the period spent at low temperature was increased leads to the same conclusion. Eggs in which the diapause was least intense were able to complete their diapause development in a very short time at low temperature whereas those in which diapause was more intense required a longer period at low temperature to allow of successful completion of their diapause development. Diapause having been completed at low temperature, eggs were able to make full use of the high temperature of incubation and resume their morphogenesis immediately.

Diapause was, however, completed by some eggs even when maintained at constant high temperature, since some eggs hatched under these conditions, but a much longer time was required than when diapause development proceeded at low temperature, and a much lower proportion of eggs were able to complete their development to the point of hatching. Andrewartha (1943) showed that certain low temperatures were more influential in promoting the completion of diapause development than others and the same is probably true of *Gryllulus*, 26.8°C. being near the upper end of the range of effective low temperatures. Work at present in progress confirms this.

Preliminary high temperature treatment for more than two days caused the eggs to enter diapause more firmly than they did if subject to little or no high temperature treatment prior to their treatment at low temperature. Such eggs required a much longer exposure to low temperature to permit them to complete their diapause development than eggs that had not been exposed to high temperature initially. This can be seen by comparing the hatching distributions of treatments 0-30 and 2-30 with those of 6-30, 6-45, and 6-60 (Fig. 1), when it is clear that the distribution of hatching following treatment 6-60 was very similar to those of treatments 0-30 and 2-30, whereas there was a progressive diminution in the number of eggs hatching and increase in the variance of the hatching distributions as the period spent at low temperature became shorter. This cannot have been due to any detrimental influence of the preliminary exposure to high temperature *per se*, but must rather have been due to the insufficiently long period of exposure to low temperature. But since 30 days was an adequate exposure to low temperature to enable diapause development to be completed following little or no preliminary high temperature treatment, it follows that the diapause in the eggs given a lengthy initial exposure to high temperature was more intense and so required a longer period for its completion.

High temperature then influenced the eggs of *Gryllulus* in two ways. Initially eggs were induced to enter diapause more firmly under the influence of high temperature than when no preliminary high temperature treatment was given, but diapause was completed in some cases at least whilst the eggs remained at high temperature. This double influence of high temperature is most satisfactorily explained on the assumption that the physiological processes concerned in the inception of diapause in the eggs and those occurring during

diapause (diapause development) have different temperature optima but that the ranges of temperature tolerable to each group of processes overlap to some extent. Very low temperatures (12.8°C.) then would be near the optimum for diapause development but would not be influential in increasing the intensity of diapause whereas high temperatures (26.8°C.) tend to increase the intensity of diapause but at the same time they permit the processes of diapause development to continue, though slowly and inefficiently.

It has been shown in Section III(*e*) that some 20 per cent. of the eggs laid under the conditions of this experiment were without a diapause stage in their development (Fig. 2), but the experiment gives no information about the conditions under which such eggs occur. It is not known, for example, whether the diapause-free eggs were laid by females laying only such eggs or whether most females laid some. Work at present in progress indicates that the temperature of incubation influences the percentage of eggs that enter diapause, and Simmonds (1948) has shown that in certain Hymenoptera the physiological state of the female is important in influencing the incidence of diapause in her progeny. Some such condition may have been operating in this case.

Diapause in the eggs of Orthoptera has been shown to occur at various stages in the morphological development of the embryo in different species. In *Austroicetes cruciata* the embryo is in a very early stage of development with little morphological differentiation when diapause occurs (Andrewartha 1943), whilst in *Melanoplus differentialis* diapause occurs at the stage when revolution round the posterior pole of the egg is about to begin (Slifer 1932). Again Moore (1948) and Salt (1949) have shown that in *Melanoplus bivattatus* and *M. mexicanus* diapause occurs at a stage when the embryo has almost completed its morphological development. In *G. commodus* it was found that, in eggs that had remained for about 20 days at 26.8°C. without evident signs of development, the embryos when fixed and stained were for the most part in the stage where katatrepsis had commenced but before the embryo had reached the stage where revolution occurs. This stage is reached by about the fourth day of incubation by eggs that had completed their diapause at low temperature with no preliminary high temperature treatment. This may account for the observation that two days preliminary high temperature treatment had no detrimental influence on the subsequent completion of diapause in the eggs whereas six days high temperature treatment considerably interfered with development at low temperature. It would seem that the egg was able to make full use of the low temperature period provided this occurred before embryonic development had proceeded beyond the point where diapause normally occurred, whereas if the low temperature treatment was given later than this diapause development proceeded less efficiently. It was notable that in eggs that had not hatched even after two months at constant high temperature, the embryos were frequently malformed. On two occasions embryos were found to have almost completed their development without having undergone revolution. Andrewartha (1943) reported similar "monsters" in *Austroicetes* that had been kept continuously at high temperature.

In Section III(*f*) it was noted that certain eggs in treatments 12-30, 12-45, and 12-60 were black on removal from low temperature. Dissections of these eggs showed that the embryos were fully developed and darkened, whereas in the young nymph darkening of the cuticle does not visibly begin at 26.8°C. until about an hour after hatching. It has been shown in the eggs of *Melanoplus differentialis* that tyrosinase activity rose steadily for the first 20 days of development and then remained fairly constant (Bodine and Boell 1935). The enzyme was almost entirely confined to the yolk and serosal cells until the time of yolk engulfment when activity appeared in the embryo. Dennell (1947) has shown that in the larva of *Sarcophaga falculata* tyrosine was present during the rise in tyrosinase activity but that the two were prevented from reacting by a low oxidation-reduction potential. At the time of pupation there was a marked rise in the oxidation-reduction potential followed by darkening and hardening of the cuticle. Probably some mechanism very akin to these occurs in the diapause-free eggs of *G. commodus* on about the twelfth day of incubation at high temperature. Subsequent low temperature treatment no doubt slowed down the rate of darkening and hardening of the cuticle without completely inhibiting it, with the result that at the end of about 30 days some of the embryos were black and hard. Hardening of the cuticle would then inhibit the action of the cephalic vesicle, which assists in hatching (Cappe de Baillon 1920).

It was also noted in Section III(*f*) that in the above three treatments some nymphs were found that had hatched whilst at low temperature. Slifer (1938) has shown that in *Melanoplus differentialis* hatching is accomplished by means of an enzyme (or complex of enzymes), secreted by the pleuropodia just before hatching is due to occur. These organs are also present on *Gryllulus* embryos and doubtless subserve the same function. It seems likely then that at about the time the eggs were moved to low temperature after 12 days at high temperature, the hatching enzyme was secreted by embryos that were in an advanced stage of development owing to the absence of a diapause stage and that the action of this enzyme, although retarded at low temperature, was not inhibited completely, with the result that after about 30 days some nymphs were able to hatch. All such nymphs were black, none had succeeded in escaping from the hatching membrane, and all were dead.

It may be said of both of the above phenomena that initiation need not have occurred at high temperature but rather after removal to low temperature. This may be so, but in view of the late stage of morphological development such embryos had reached after 12 days at high temperature it is likely that such processes would have come into operation by that time. In any case the principle remains unaltered that development, in the sense of the continuation of these two processes at least, was able to continue to completion at a slow but still perceptible rate at low temperature, whereas it seems likely that development in the sense of morphological differentiation was slowed to an extremely low rate at low temperature.

V. Acknowledgments

The author wishes to thank Mr. D. C. Swan, Head of the Department of Entomology, Waite Agricultural Research Institute, for his constant interest and encouragement during the course of this work. To Dr. H. G. Andrewartha, also of this Department, is owed a debt of gratitude for his kindly help and discussion on all phases of this work. Thanks are also due to Dr. E. A. Cornish, Section of Mathematical Statistics, C.S.I.R.O., for guidance in the statistical treatments used. Miss M. E. Morphett gave valuable technical assistance and prepared the figures.

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OBSERVATIONS ON THE FEEDING OF THE VIRUS VECTOR *OROSIUS ARGENTATUS* (EVANS), AND COMPARISONS WITH CERTAIN OTHER JASSIDS

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Summary

Examination of feeding tracks of several species of jassids in plant petioles shows that each species produces characteristic stylet sheaths. Most species feed in both parenchyma and vascular bundles and they exhibit varying degrees of preference for phloem tissue. An attempt to confirm published observations on a pH gradient towards the phloem failed to reveal such a gradient in many of the plants examined; in fact, it has been demonstrated that no such gradient is required to explain the feeding behaviour of the jassids studied. It is concluded that they find the tissues upon which they feed by random probing with the stylets.

A study of the details of jassid feeding has suggested possible explanations for several puzzling observations on virus transmission by these insects.

I. Introduction

The feeding processes of an insect vector of a virus disease are of obvious significance, for it is during these events that the pathogen is first taken up from one host and subsequently reinoculated into a second susceptible host. The common brown jassid *Orosius argentatus* (Evans) is a vector of several phytopathogenic viruses of importance in Australia; but very little is known of its physiology. This deficiency is the more regrettable because generalizations on jassid feeding have been largely based on the observations made on *Circulifer* (*Eutettix*) *tenellus* (Baker), the vector of curly top of sugar beet (see, for example, Bennett 1935; Bennett and Wallace 1938), and on *Cicadulina mbila* (Naude), vector of maize streak (Storey 1938, and earlier papers quoted therein). That these two species, both mainly phloem feeders, are not representative of the range of vector behaviour has recently been strikingly demonstrated by the discovery that all the vectors of Pierce's disease of grape feed mainly on the xylem (Houston, Esau, and Hewitt 1947). The mechanism whereby *Circulifer tenellus* finds the phloem of the sugar beet has been studied in an ingenious series of experiments by Fife and Frampton (1936). They reported a gradient of increasing alkalinity from the cortex to the vascular bundle amounting to a difference of about 1.6 pH units. Further, they found by feeding an artificial diet that the insects could distinguish between media of different pH and that they preferred an alkaline medium; they con-

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cluded that the jassids find the preferred tissue of the host with the aid of this pH gradient. It has been assumed that this mechanism holds for other jassids but confirmatory evidence has not been published. It is the purpose of this paper, therefore, (1) to report observations on the plant tissues upon which *O. argentatus* and several other jassids feed; (2) to consider the methods by which they find these tissues with reference to the Fife and Frampton pH gradient hypothesis; and (3) to describe observations on mouth-part movements and related details that assist in giving an outline of the feeding behaviour of some vectors and non-vectors of phytopathogenic viruses. All of the jassids were from laboratory colonies originating from insects collected in the vicinity of Canberra, usually by trailing nets from a jeep traversing stands of lucerne.

II. The Tissues on which Several Species of Jassids Feed

(*a*) *Characteristics of Feeding Tracks of Six Species of Jassids*

Jassid feeding tracks are made readily visible in sections of plant tissues by the use of Millon's reagent for tyrosine (Smith 1933). Many sections of plant petioles, stems, or leaves have been cut approximately 150 μ in thickness in a hand microtome and immediately placed in Millon's reagent. After about 10 minutes, jassid feeding tracks are reddish and can be clearly distinguished from the plant tissues. Evidence that the reaction gives an accurate picture of the position of the insect mouth-parts in the plant is given below (Section V). The sections were examined with a dissecting microscope under a magnification of about 40 diameters, and the tracks drawn with the aid of a camera lucida. Numbers of tracks can be found if many insects are exposed for several days on a few petioles, or better, on a restricted portion of a single petiole.

Table 1

TISSUES OF *MALVA PARVIFLORA* PETIOLES IN WHICH FEEDING TRACKS OF SEVERAL JASSIDS TERMINATE

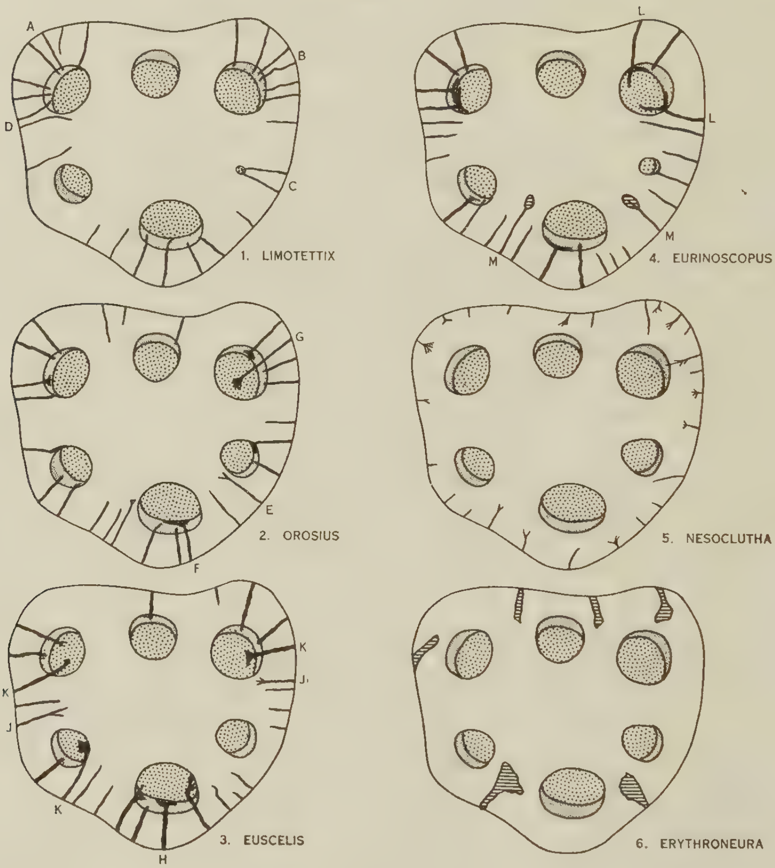
Species	Total Tracks Counted	Percentage of Tracks in		
		Phloem	Xylem	Parenchyma
Limotettix sp.	109	63	0	37
Orosius argentatus (Evans)	108	54	3	43
Eurinoscopus punctatus Evans	104	37	8	55
Euscelis sp.	128	37	27	36
Nesoclutha obscura (Evans)	77	5	0	95
Erythroneura ix Myers	20*	5	5	90

* This species did not feed readily on *Malva* and hence it did not prove feasible to obtain a greater number of tracks.

In order to study the tissues preferred for feeding, petioles or stems with collateral vascular bundles without secondary thickening are necessary. Those of the petiole of *Malva parviflora* (L.) have proven very suitable. The results, showing the final tissue reached by several species of jassids, are given in Table 1.

From these data it may be concluded that (1) it is usual to find some feeding tracks from any one species terminating either in vascular bundles or in parenchyma, and (2) species vary in respect to the plant tissues upon which they generally feed.

The second conclusion presupposes some ability of the insect to distinguish tissues. It also suggests that the species studied would vary in their ability to transmit phloem viruses on this characteristic alone. More detailed information was gained from a study of the form of the tracks made by each species.



Figs. 1-6.—Diagrams of transverse sections of petioles of *Malva* showing feeding tracks of (1) *Limotettix,* (2) *Orosius,* (3) *Euscelis,* (4) *Eurinoscopus,* (5) *Nesoclutha,* (6) *Erythroneura.* For further details see text.

Diagrams of random sections of *Malva* petioles containing superimposed feeding tracks are presented in Figures 1-6. The following conclusions with respect to each species may be drawn from the data presented in these figures.

(1) *Limotettix.* Not only is there a high percentage of the tracks that terminate in the phloem tissue, but it will be observed that they are concentrated in regions where they will reach the phloem without curving or branch-

ing (Fig. 1, *A* and *B*). There is an absence of tracks from the lateral regions of the petiole, except at points such as *C*, in which example the tracks run almost directly to a very small vascular bundle. There is little curving of the tracks, but at *D* there are two tracks that curve slightly, one in the direction of the phloem, the other away from the vascular bundle.

(2) *Orosius.* The percentage of tracks reaching the phloem is lower than in the previous species, and the tracks are more uniformly distributed around the petiole. Branching occurs in some tracks (*E*) and some slight tissue damage is apparent, especially in those tracks that terminate in the phloem (*F*). These are noticeably larger than tracks terminating in the parenchyma, probably owing to the insect feeding for a longer time on phloem than it does on parenchyma. Occasional tracks terminate in the xylem (*G*).

(3) *Euscelis.* This species produces more tissue damage (*H*) than the damage following feeding by *Orosius.* More tracks terminate in the xylem (*K*), and branching is more frequent (*J*). The tracks are generally larger than those of *Orosius*, probably correlated with the larger size of the jassid.

(4) *Eurinoscopus.* The characteristics mentioned for *Eucselis* are still more marked in *Eurinoscopus.* Tissue damage is more severe and may occur in parenchyma (*M*). A number of tracks run both to phloem and xylem (*L*), but some tracks terminating in phloem are quite simple and apparently do not cause much tissue damage.

(5) and (6). Reference to the figures of *Erythroneura* and *Nesoclutha* feeding tracks will illustrate that these species cannot be effective vectors of phloem-restricted viruses.

(b) Differences in Feeding Tracks in Different Plant Organs, Plant Species, etc.

Feeding tracks of some of the same insects were studied in other plants and in leaves. Similarly the tracks of *Orosius* nymphs were studied to compare them with those produced by adults. The results are presented in Table 2.

These data when compared with those in Table 1 show that the feeding tracks of a single species terminate in the same tissues in varying frequencies depending upon the species of plant. Thus, *O. argentatus* tracks terminate in the phloem in 54 per cent. of cases on *Malva,* in 48 per cent. of cases on *Beta,* and in 2 per cent. of cases on *Datura,* when the petiole is almost too large to permit the stylets reaching the phloem. (The longest track left by *Orosius* was found in such a *Datura* petiole and measured about 500 μ in length.) Similarly, *Eurinoscopus* reached the phloem on 37 per cent. of occasions in *Malva* but on 50 per cent. of occasions on lucerne, in which the phloem occupies the major portion of the circumference of the stem at the depth to which the stylets of the insect can penetrate.

When feeding on the leaf the jassids will almost invariably reach the phloem if they feed on the under surface of the leaf veins. They do, in fact, appear to have a preference for such situations, as was indicated by the observations upon feeding on the *B. vulgaris* leaf (Table 2). The feeding tracks

of nymphal instars of *Orosius* produced tracks averaging only slightly shorter than those of the adults (respectively 230 μ and 270 μ) in large *Datura* petioles but otherwise were similar.

TABLE 2

TISSUES IN WHICH FEEDING TRACKS OF JASSIDS TERMINATE

Jassid	Host Plant and Tissue	Total Tracks Counted	Percentage of Tracks in		
			Phloem	Xylem	Parenchyma or Mesenchyma
O. argentatus adults	*Beta vulgaris* petiole	114	48	4	48
O. argentatus adults	Large *Datura stramonium* petiole	53	2	1	97
O. argentatus 1st-3rd instar	*B. vulgaris* petiole	112	23*		77
O. argentatus 4th instar	*B. vulgaris* petiole	106	18*		82
O. argentatus adults	*B. vulgaris* leaf	55	29	2	69
E. punctatus adults	*Medicago sativa* stems	54	50	24	26

* Most of these tracks terminated in the very small bundles in the "wings" of these petioles, which were unusually large, and in these bundles it was impossible to determine precisely where the insect was feeding.

(*c*) *Ability of the Jassids to Distinguish the Tissues of Plants*

The following is a summary of observations that together suggest strongly that some jassids can distinguish tissues of plants upon which they feed. (1) The feeding tracks of *Orosius* are frequently larger when they terminate in the phloem than when they terminate in the xylem. (2) *Limotettix* certainly feeds more frequently on phloem than on other tissues. (3) The frequency of tracks curving towards the phloem is greater than the frequency of those curving away from it (Figs. 1-6). (4) Observations on the behaviour of *Limotettix, Orosius,* and *Euscelis* show that a preponderance of the insects feed on the veins on the ventral surface of leaves in preference to other parts of the plant.

The conclusion seems justified that some species of jassids show a preference for feeding on the phloem tissue.

III. AN INVESTIGATION OF THE pH GRADIENT HYPOTHESIS

If, as has been shown in the previous section, some jassids show a preference for feeding on phloem tissues, the next question concerns the method by which they recognize or locate this tissue. Fife and Frampton (1936)

suggested the attractive hypothesis that *Circulifer* (*Eutettix*) *tenellus* finds the phloem of the sugar beet by a pH gradient from the parenchyma towards the phloem. It seemed possible that differences in infectivity rates in crops attacked by *Orosius argentatus* might be explained by the varying ability of this jassid to locate the tissues in each crop in which the virus could multiply, and it was therefore decided to repeat Fife and Frampton's experiment on several host plants of *Orosius*. Gradients of pH from the epidermis to the phloem were sometimes found in some of these plants, but not in others. The gradients when present were generally much less marked than those reported by Fife and Frampton. The evidence for these conclusions is given in the remainder of this section.

TABLE 3

pH MEASUREMENTS IN PETIOLES OF SUGAR BEET PLANTS GROWN UNDER SIMILAR GREENHOUSE CONDITIONS. TWO PETIOLES FROM EACH PLANT

Tissue	Plant 1		Plant 2		Plant 3		Plant 4		Plant 5	
Parenchyma	5.6	5.9	5.5	5.6	5.8	5.8	5.7	5.9	6.0	6.1
Parenchyma	5.6	5.8	5.6	5.7	—	—	—	—	—	—
Mech. tissue	5.7	5.7	5.9	5.7	—	—	—	—	—	—
Phloem	5.7	5.8	5.8	5.7	6.1	6.1	6.2	6.2	6.8	6.3
Xylem	5.9	5.9	5.6	5.9	—	—	—	—	—	—

(*a*) *Methods*

The method described by Fife and Frampton employs a modified quinhydrone electrode and is open to a number of objections. The quinhydrone method gives low readings and is not accurate above pH 8.0. It is liable to protein errors and is not suitable in the presence of oxidizing or reducing agents. In addition, the micro-scale modification suffers from uncertainties due to quinhydrone solubility in cell sap, tissue damage in the phloem even by the 40 μ diameter electrodes, and the effect of the saturated KCl on the plant tissues. Nevertheless, the method was found to be the most suitable of any used and an attempt has been made to follow the technique of Fife and Frampton as closely as possible. Platinum needles were soldered to short, shielded leads and sealed in glass tubes. They were tested on a series of buffers from pH 4.0 to 8.0, saturated with quinhydrone. Only electrodes that responded over this range with an accuracy of $\pm$ 0.1 pH unit were used. The Cambridge bench-type pH meter used was standardized before and after each series of tests on a buffer solution at pH 4.0 (0.05M phthalate) and checked on a buffer solution of pH 8.0 (0.067M phosphate). Special precautions were taken with leads, junctions, earthing, shielding, bridges, cleaning of electrodes, and similar details.

Much the same results were obtained when the electrical connection with the standard calomel electrode was made through the length of the petiole, as in Fife and Frampton's work (in which the resistance between the electrodes approximated 100,000 ohms), or when this connection was achieved by means

of an asbestos fibre sealed in a glass capillary filled with saturated KCl solution and immersed in the cell sap on the cut surface of the petiole a few cells from the platinum electrode (Fig. 7). Resistance between the electrodes using this method approximated 70,000 ohms. The pH meter was capable of measuring potentials in systems with resistances of 100,000,000 ohms.

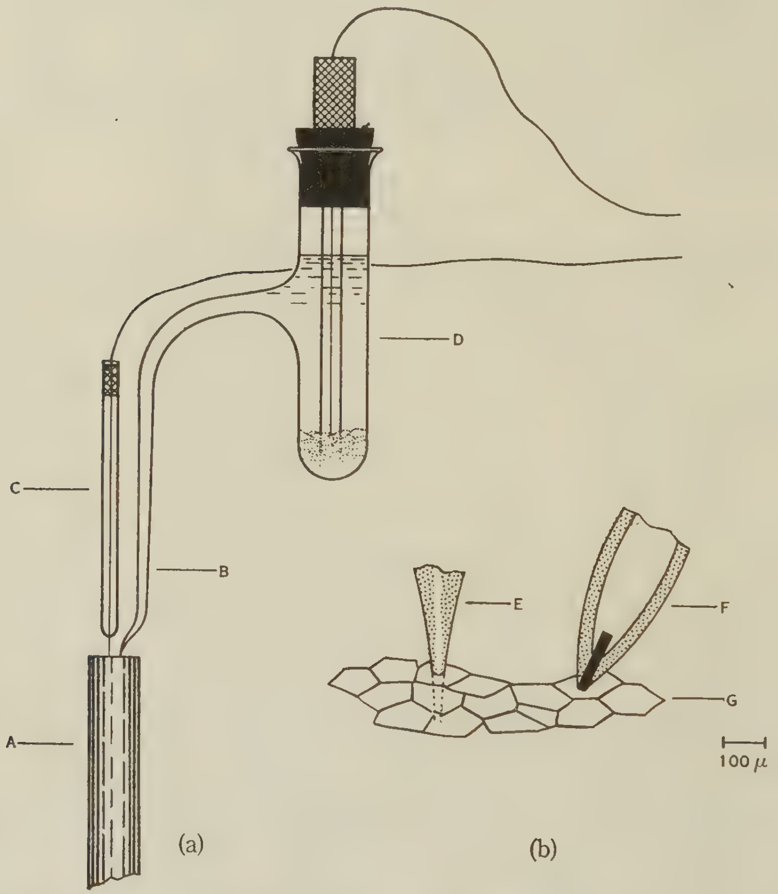


Fig. 7.—Diagram of electrode assembly for measuring pH of plant cell sap.
(*a*) Calomel half cell and platinum electrode applied to the cut surface of a petiole.
(*b*) Detail of immersion of electrodes in cell sap.
A, petiole; *B*, KCl bridge with asbestos fibre sealed in a capillary tip; *C*, platinum electrode; *D*, calomel half cell; *E*, platinum needle; *F*, tip of KCl bridge with asbestos fibre; *G*, cut surface of a petiole.

(*b*) *Results*

A number of readings with this apparatus have been made. In Tables 3-6 inclusive the vertical columns represent a series of pH readings progressing from the epidermis towards the vascular bundles. Table 3 gives the pH

TABLE 4

pH MEASUREMENTS IN PETIOLES OF SUGAR BEET PLANTS GROWN UNDER DIFFERENT CONDITIONS

A, greenhouse; *B*, outdoors

	A_1		A_2		A_3		A_4		B_1		B_2		B_3		B_4	
Parenchyma	5.6	5.4	—	—	—	—	—	—	6.1	6.0	5.6	6.0	6.0	5.9	5.7	5.6
Parenchyma	5.9	5.6	5.9	5.6	5.9	5.9	6.1	6.1	6.1	5.8	5.9	6.1	6.5	6.3	5.9	5.7
Parenchyma	5.7	5.6	5.8	5.9					6.1	6.0	6.2	6.3	6.8	6.5	6.1	6.0
Mech. tissue	5.7	5.7	5.7	5.8					6.1	6.2	5.9	6.4	7.3	7.3	6.3	6.2
Phloem	5.8	5.7	5.7	5.9	6.2	6.1	6.0	6.1	6.2	6.5	6.3	6.7	7.3	7.8	6.2	6.5
Xylem	5.8	5.9	5.8	6.0					5.9	6.1	6.1	6.6	7.0	7.2	5.8	5.8

TABLE 5

pH MEASUREMENTS IN PETIOLES OF FOUR SUGAR BEET PLANTS

A—a normal plant from greenhouse
B—the same plant from greenhouse; petioles upon which *Nezara* had fed
C—virus (Big Bud) infected plant from greenhouse
D—infected plant from outdoors

	A				*B*		*C*		*D*					
Tissue	16.ii.51		21.ii.51		21.ii.51		19.ii.51		14.ii.51		15.ii.51		19.ii.51	
Parenchyma	5.6	5.4	5.6	5.8	5.6	5.5	5.6	5.6	6.0	5.9	6.3	6.3	5.6	5.7
Parenchyma	5.9	5.6	5.8	5.8	5.7	5.7	5.7	5.9	6.5	6.3	6.1	6.0		
Parenchyma	5.7	5.6	—	—	—	—	—	—	6.8	6.5	6.4	6.2		
Mech. tissue	5.7	6.7	5.8	5.8	5.8	5.8	5.9	6.0	7.3	7.3	6.7	6.4		
Phloem	5.8	5.7	5.8	5.7	6.0	5.9	6.0	6.3	7.3	7.8	7.4	7.1	6.5	6.6
Xylem	5.8	5.9	5.8	5.7	5.9	5.8	5.7	5.9	7.0	7.2	5.7	5.5		

measurements on a series of sugar beet petioles grown under practically identical conditions in the greenhouse. They indicate a plant to plant variation in pH of the different tissues (greatest range in phloem pH 5.7 to 6.8); and even between two petioles from the same plant substantial differences in pH exist (greatest range in phloem pH 6.3 to 6.8). More important, it will be noted that in this series the maximum difference (in plant No. 5) between parenchyma and phloem is about 0.8 pH units. In most the difference is much smaller than this, and the same applies to the bulk of the data presented in this section, whereas Fife and Frampton report a range of 1.6 pH units or more.

Table 4 presents similar data for two series of plants, the first grown in the greenhouse and the second grown outdoors. The conclusion seems justified that the results in the second series are higher than those in the first, but in neither is there always a progressive pH gradient from parenchyma to phloem, although in some of the plants grown outdoors such a gradient undoubtedly occurs. On the contrary in plant A_4 the pH of the parenchyma is almost the same as that in the phloem.

In Table 5 data are presented which indicate that the pH of petioles from the same plants may change from day to day. The data (column B) also suggest that the feeding of certain Hemiptera on a petiole does not in itself result in pH changes and that infection by the virus of Big Bud of tomato in the beets did not cause marked changes in pH (compare column D with plants $B_{1\text{-}4}$ in Table 4).

Table 6

pH MEASUREMENTS IN PETIOLES FROM DIFFERENT SPECIES OF PLANTS

Tissue	Pumpkin		Tomato		Tobacco	
Parenchyma	5.9	5.9	5.7	6.1	5.5	5.4
Parenchyma	5.8	5.9	5.9	6.1	5.5	5.4
Parenchyma	6.1	5.8	6.1	6.1	5.5	5.4
Phloem	6.4	6.4	6.2	6.2	5.9	6.0
Xylem	6.4	6.3	6.2	6.2	5.8	6.3

Table 6 gives representative data showing that the conditions in sugar beet are found also in other species. In some petioles pH gradients were observed, in others, no such gradients. In pumpkins it was regularly observed that the tissues of the vascular bundles were less acid than the parenchymatous tissue. However, the pH of the phloem and of the xylem were not greatly different in most petioles in cucurbits.

(c) *Critique of the Hypothesis of pH Gradient as a Factor in Assisting Jassids in Finding the Phloem*

A consideration of the following points suggests that no gradient of any kind assists the jassids studied in locating the tissues on which they prefer to feed.

(1) Observations on the feeding tracks referred to in Section II(*b*) do not suggest that the insects were responding to a gradient. The number of straight tracks greatly exceeds the number that curve in the species studied.

(2) *Orosius* adults were fed on young leaves of pumpkin. Feeding tracks did not terminate in the vascular bundles with any greater frequency than in plants that did not have alkaline vascular bundles. Similarly, *Orosius* were fed on *Beta* plants differing in the pH of their tissues (plants B_1 and B_3 of Table 4). No difference was found in the distribution of feeding tracks in these two plants.

(3) The data of Day and McKinnon (1951, Table 8) on the amount of material ingested of pH from 4.2 to 9.0 lends no support to the theory that *Orosius* reacts to the alkalinity of its food.

(4) Examination of the data presented by Fife and Frampton (1936, Table 3) and our Tables 1 and 7 suggests that the total number of insects reaching the phloem in their work was not much greater than those in our experiments. Admittedly it is not easy to make a direct comparison because of differences in the methods of presenting the data, but with *Circulifer* on sugar beet, about 55.8 per cent. reached the phloem compared with *Orosius* in which 48 per cent. of the feeding tracks terminated in the phloem.

IV. How do the Jassids Find the Tissues upon which They Prefer to Feed?

In the previous section evidence has been presented that certain jassids at least do not utilize a pH gradient to find the phloem; no alternative hypothesis has previously been suggested. Data substantiating such an alternative will now be given.

Examination of Table 1 and Figures 1-6 indicates that the species studied fall into three groups:

(*a*) The species of *Erythroneura* and *Nesoclutha* are evidently primarily parenchyma feeders, as were the majority of species of *Empoasca* studied by Smith and Poos (1931), and the jassids described by Smith (1926).

(*b*) In the second group of jassids are included species whose feeding tracks reach the phloem in about 30-50 per cent. of cases, i.e. the species of *Orosius, Eurinoscopus,* and *Euscelis.* These can distinguish the phloem when they reach it, but apparently feed also on the parenchyma. The principal evidence for the former statement comes from the observation that the tracks that terminate in the phloem are usually considerably larger than those in the parenchyma. Evidence suggesting that these species feed on the parenchyma comes from two observations: (i) *Orosius* have been shown by Day and McKinnon (1951) to feed on a variety of liquids, including distilled water (containing tracer quantities of ^{32}P); (ii) they live for some days when provided only with delaminated stems of *Datura* in which they are unable to reach the vascular bundles.

Measurements of the relative proportions of the circumference occupied by phloem and parenchyma at a depth to which the stylets can penetrate in

Malva and *Beta* petioles are given in Table 7. Comparison of these data with those in the last column of the table (taken from Tables 1 and 2) indicates that the percentage of feeding tracks terminating in the phloem is of the order of magnitude of the relative sections of phloem and parenchyma. Only approximate figures can be given because of the variability in different regions of a single petiole but the figures are reasonably accurate.

There is good evidence, therefore, that the species studied belonging to the genera *Orosius, Eurinoscopus,* and *Euscelis* feed on whatever tissues they reach with preliminary probing, but that they feed longer on the phloem than on the parenchyma.

(*c*) The third type of feeding behaviour is exemplified by *Limotettix.* In this, the insect clearly reaches the phloem a significantly greater number of times than it would by chance. From Figure 1 it will be observed that the number of curving feeding tracks is too low in *Limotettix* for them to account for the high percentage of tracks terminating in the phloem. This in itself is evidence that this species does not employ a gradient of any sort to assist in finding the phloem.

Table 7

COMPARISON BETWEEN APPROXIMATE SECTION OF PLANT TISSUES AND NUMBER OF FEEDING TRACKS TERMINATING IN PHLOEM

Species of Plant	Approximate Mean Percentage Cross Section of		Percentage of Feeding Tracks of *Orosius* Terminating in Phloem
	Parenchyma	Phloem	
Beta	50	50	48
Malva	40	60	54

Examination of Figure 1 (especially the tracks at *C*) suggests that *Limotettix* must either detect the vascular bundle from the exterior of the petiole or that, in probing, it generally leaves no feeding tracks unless it reaches the phloem. The first hypothesis is difficult to accept but direct evidence for the second results from a comparison of measurements made of the time *Orosius* and *Limotettix* spend in feeding on the petioles of *Malva.* It was found that 36 per cent. of the *Orosius* had feeds of less than 4 minutes duration whereas 80 per cent. of the *Limotettix* fed for less than 4 minutes. It was obvious that *Limotettix* was more restless than *Orosius,* and its normal behaviour when alighting on a *Malva* petiole is for it to probe two or three times before settling to feed, whereas *Orosius* normally feeds the first time it probes the plant tissues. It seems very likely that *Limotettix* leaves no feeding tracks in its preliminary probings.

These data suggest that the tissues reached by the stylets of *Limotettix* depend solely upon chance, and thus bring this species into line with those considered in the first two groups.

Fife and Frampton (1936) present some data (their Table 3) that require an explanation, namely the reduction in the number of feeding tracks of *Circulifer* that terminated in the phloem after CO_2 treatment. If the mean percentage cross section of phloem approached that of the petioles used in the present investigation it is difficult to understand how only 12.12 per cent. of feeding tracks could have terminated in the phloem unless the insects were actually repelled by that tissue. The greatest increase in the number of feeding tracks in the CO_2-treated plants is in the group in which the punctures "were not in direction to hit phloem and not deep enough." It is possible that the treatment made the tissues unpalatable, but in view of the report that the amount ingested by Orosius was not greatly influenced by pH (Day and McKinnon 1951), this explanation does not seem likely. Further work on this problem raised by Fife and Frampton's observation is clearly needed.

V. Movements of the Mouth-Parts, Stylet Sheath Formation, etc.

The penetration of plant tissues by the mouth-parts cannot be observed directly, but there is no reason to believe that the mechanism differs from that which can be observed when the insect is induced to puncture a transparent plastic membrane as described by Day and McKinnon (1951). The insect braces its prothoracic and mesothoracic legs, rotates the head so as to bring the rostrum (which normally is directed posteriorly between the prothoracic legs) perpendicular to the surface to be penetrated. The surface of a plastic membrane is actually dented by the pressure of the mouth-parts and almost immediately the feeding stylets, comprising the mandibles and maxillae, appear through the membrane, and extend about 70 μ into the fluid. The insect may then either withdraw the stylets and move off, leaving no trace of its presence, or settle to feed. In the latter event it proceeds to form a salivary sheath. The stylets perform a fairly rapid oscillating motion. As they are withdrawn a small particle appears at the tip. This is shaped into a tube-like sheath by the next forward movement. Each movement adds to the length of the sheath at its distal end (although sometimes the sheath forks at the tip). It reaches a length of about 70 μ in a minute or so, but may be added to at any time subsequently during feeding. The stylets then extend beyond the sheath (sometimes for brief periods to more than three times its length) and are moved to and fro in the fluid. This probably occurs during actual ingestion. Withdrawal of the mouth-parts from the sheath occurs after feeding is complete or if the insect is disturbed. It may be effected with considerable speed and without difficulty. The hind legs are placed on the membrane and the insect withdraws the stylets and moves away. If the disturbance is caused by another insect or change of light intensity, the stylets may be reinserted in a position close to the previous one, often without any other movement of the insect.

No important differences have been observed between any of the species studied; except that *Nesoclutha* and *Erythroneura* insert their mouth-parts at an

angle of about 45° with the membrane, whereas those of the other species usually do so perpendicular to it. The above description also agrees with that given by Storey (1939) for *Cicadulina mbila* feeding through a paraffin membrane, and we differ from Storey only with reference to the occurrence of material other than the salivary sheath flowing from the stylets. The observations were not conclusive on this point but we have visual evidence of the existence of such a substance.

The above observations give support to the conclusion that the insects feed at the termination of the feeding tracks as determined by Millon's reagent. The tracks are longer in plant tissues than they are in a fluid medium but there is no need in the fluid for longer tracks although, as mentioned above, occasional exploratory extensions of the stylets may occur.

VI. Discussion and Conclusions

It has often been observed by workers on jassid transmission of plant viruses that a jassid infects only some of a series of plants to which it is exposed. Bawden (1950, p. 87) considers it probable that such results indicate "that the salivary glands are easily exhausted of virus and that there is not a steady flow of virus from the blood to the glands, but that it takes place irregularly." It is difficult to conceive of a physiological mechanism that would account for varying rates of flow of the virus into the salivary glands. The results could, however, be explained on the basis of observations reported above, namely that the jassid may not reach the viruliferous tissue or the tissue in which the virus can develop every time it feeds. It seems probable also that certain of the results of Storey (1938) can be explained in the same way.

For a jassid to be an efficient vector of a phloem-restricted virus at least two conditions must be fulfilled with respect to its feeding behaviour. Firstly, it must reach the phloem in a reasonably high percentage of feeds, and secondly it must presumably not cause undue damage in the phloem, which might inhibit the development of the virus. The data presented in the previous sections permit an evaluation of the species studied as virus vectors on the basis of these two criteria. It should be emphasized that factors other than feeding behaviour, such as the permeability of the gut and salivary gland to the virus, may play a significant role in determining whether a species acts as a vector. The work of Storey (1933) illustrates this with the strains of *Cicadulina mbila*. However, it is equally true that the role of the feeding behaviour has been neglected in previous work. One further qualification should be appreciated. The data of Table 1 were obtained from insects feeding on the petioles of *Malva*. Although it is suggested from other data, such as some of that in Table 2, that these figures give a guide to the feeding behaviour, it is true that a jassid will reach the phloem of many plants whenever it feeds on the underside of a small leaf vein. The opportunity for transmission of plant viruses by jassids is, therefore, greater than is indicated by the crude

numerical values in Table 1, especially when the exposure times are sufficient for several feeding periods. Our results indicate that the species studied make approximately six feeding tracks per day at room temperatures.

With these qualifications it will be clear that the ability of the insects studied to act as vectors of phloem viruses, on the basis of feeding behaviour alone, is in the following order: *Limotettix, Orosius, Euscelis, Eurinoscopus.* Neither *Erythroneura* nor *Nesoclutha* would be expected to act as vectors of phloem-restricted viruses.

Limotettix would appear to be the most efficient vector, both because of its clean feeding tracks, which cause very little tissue disturbance, and because of its ability to find the phloem in a high percentage of feeds. *Orosius* has clean feeding tracks but is significantly less efficient in finding the phloem. *Euscelis* caused more tissue damage, whereas *Eurinoscopus* caused quite severe necrosis at the sites of feeding. The amount of injury seems to increase with the size of the jassid. It is noteworthy that vectors of plant viruses are mostly small jassids. However, it is conceivable that a certain amount of tissue damage might provide an accumulation of protein (Smith 1933), which could be a suitable medium for the establishment of a virus infection. Tests are planned to determine the ability of the jassids mentioned above to act as vectors of certain plant viruses.

VII. Acknowledgments

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STUDIES ON THE DIGESTION OF WOOL BY INSECTS

IV. ABSORPTION AND ELIMINATION OF METALS BY LEPIDOPTEROUS LARVAE, WITH SPECIAL REFERENCE TO THE CLOTHES MOTH, *TINEOLA BISSELLIELLA* (HUMM.)

By D. F. WATERHOUSE*

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Summary

The fate was investigated of 30 metallic and five non-metallic elements following ingestion by *Tineola* larvae. When incorporated in woollen fabric or in a yeast-casein medium, 19 elements that form insoluble sulphides produced characteristically coloured sulphides in the food undergoing digestion in the midgut. The production of the sulphides is brought about by the alkaline, highly reducing, midgut secretions, which cause the production of sulphydryl groups by the reduction of the disulphide bonds of the cystine present in the wool. When metal is present in the diet less cystine is excreted than on a normal diet. Other sulphur-containing compounds (methionine, glutathione) also permit the formation of sulphides. Much of the sulphide formed passes down the digestive tract and is excreted. However, a certain amount forms highly dispersed colloidal solutions with the amino acids or polypeptides liberated by digestion of the food or present in the digestive secretions. These colloidal sulphides are taken up by the midgut epithelium, and granules of sulphides accumulate in the cavities of the goblet cells of the anterior and posterior regions of the midgut. Sulphides of fewer metals accumulate in the goblet cells of the middle region of the midgut. All goblet cell accumulations are eliminated during moulting, when the entire midgut epithelium is cast off and regenerated. The goblet cells of other lepidopterous larvae were also shown to accumulate some metals, although not as sulphides.

Elements incapable of forming insoluble sulphides do not lead to the formation of coloured compounds in the goblet cells of *Tineola* larvae. However, the alkaline earths are deposited as granules (mainly as phosphates), principally in the columnar cells of the anterior and posterior midgut. It is probable that small quantities of absorbed fluoride are deposited with calcium in these granules.

Tineola larvae are thus able to detoxify a wider range of metals and non-metals (many of which are ordinarily highly toxic) than most other animals.

I. INTRODUCTION

The well-known resistance of wool to attack by proteolytic enzymes and its insolubility in many of the usual protein solvents is considered to be due to its high-molecular-weight polymeric structure. An important feature of this structure is the presence of disulphide cross-linkages between the polypeptide chains of the wool protein keratin. The proteolytic enzyme complex present in the digestive tract of the clothes moth larva, which is able to digest keratin,

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is very similar to that of other insects (Powning, Day, and Irzykiewicz 1951). Peculiarities in the nature of this enzyme complex, therefore, do not appear to be adequate to explain the ability of *Tineola* larvae to live on wool. It appears that this ability is largely due to their alkaline digestive juices (pH 10) and to the unusually low oxidation-reduction potential (in the vicinity of —250 to —280 mV.) in the midgut (Linderstrøm-Lang and Duspiva 1936; Waterhouse 1952*b*). These conditions result in the production of sulphydryl groups from the disulphide linkages. Wool in which the disulphide linkages have been reduced *in vitro* is attacked by proteolytic enzymes, as is also the root portion of the wool fibre, where much of the sulphur is present as sulphydryl groups as yet unoxidized to disulphide linkages (Geiger and Harris 1942; Geiger *et al* 1941).

When chemically reduced wool is reoxidized the disulphide linkages are reformed from the sulphydryl groups and the wool regains its original stability. If the reduced wool is treated with an aliphatic dihalide, pairs of sulphur atoms are linked through short hydrocarbon chains thus

$$\text{W–S–S–W} \xrightarrow[\text{agent}]{\text{reducing}} \text{W–SH} + \text{HS–W}$$

$$\text{W–SH} + \text{HS–W} + (\text{CH}_2)_n\text{X}_2 \longrightarrow \text{W–S–}(\text{CH}_2)_n\text{–S–W} + 2\text{HX},$$

where W represents the portions of the wool unconnected by the disulphide groups and X a halogen atom. When trimethylene dibromide is used for the reaction, so that W–S–$(CH_2)_3$–S–W linkages are formed, the modified wool is decidedly more stable than untreated wool to chemical agents, and furthermore it is attacked much less readily by clothes moth or carpet beetle larvae. As an example of the degree of protection conferred, wool in which the cystine content had been lowered to 6.5 per cent. suffered only 2 per cent. of the loss of weight suffered by control wool with a cystine content of 12.2 per cent. (Geiger, Kobayashi, and Harris 1942).

Linkage rebuilding in reduced wool can also be achieved by the use of metal salts, which are thought to produce W–S–metal–S–W linkages (Farnworth, Neish, and Speakman 1949; Stoves 1942). If a toxic material, such as a mercury salt, is used the resulting modified wool might be expected either to be indigestible or, if digested, with consequent rupture of metal sulphur linkages, to be highly toxic to clothes moth larvae. When wool containing mercury was fed to *Tineola* larvae the colourless fibres blackened in the middle region of the midgut and the faeces were black. This darkening was considered to be due to the liberation of mercury following rupture of the –S–Hg–S– linkages (Day 1951).

A surprising feature of these tests was that the larvae did not appear to be affected adversely by their diet. It therefore appeared to be of considerable interest to examine in *Tineola* larvae various aspects of the metabolism of those metals that might be incorporated in the chemical structure of wool as toxic agents.

The present account deals mainly with the fate of a number of metals and non-metals following ingestion by clothes moth larvae and provides data on the mechanisms whereby many toxic metal ions are detoxified by the larvae.

II. Methods

Larvae of *Tineola bisselliella* were fed at 30°C. on a standard woollen fabric, on silk, or on a yeast-casein mixture, to which diets salts of various elements, on occasion, were added. The treated fabrics were prepared in two ways. In the first method the fabric was dipped in alcohol to ensure complete wetting, washed in distilled water, immersed in a solution of an appropriate salt (various concentrations up to 20 per cent. were used) and subsequently air-dried. Those metals the salts of which form insoluble compounds with water were added in acid solution and the acids removed *in vacuo* in the presence of alkali. In the second method a number of metals were incorporated chemically in the fibre, excess metal being removed by washing in running water. The form of linkage of these metals is not at present known although reaction with acid side-chains probably occurs (Lipson, personal communication). Mercury (up to 25 per cent.), and on one occasion lead (5.0 per cent.), was added by the method of Farnworth, Neish, and Speakman (1949) mentioned earlier. Salts were added to the artificial diet either in aqueous solution or as fine powders, which were ground into the food material.

After feeding for several days on the treated food the larvae were dissected under saline and examined fresh or after fixation in 10 per cent. neutral formalin in 70 per cent. alcohol or in alcoholic Bouin. Tests specific for the metals added to the food were also applied to the fixed larvae, details of the procedures being obtained from the B.D.H. Book of Organic Reagents (1948), from Feigl (1947), and from references contained therein.

Sections were prepared in the usual manner, counterstained with eosin or aniline blue, and mounted in "Lustrex"* in mesitylene. This polystyrene mounting medium has the advantage over balsam of inertness so that, for example, the Prussian blue colour characteristic of iron does not fade (Lillie, Windle, and Zirkle 1950).

III. Results

(*a*) *Histology of the Midgut of* Tineola *Larvae*

The histology of the larval midgut has been investigated in some detail (Lotmar 1941; Waterhouse 1952*a*). There are three regions distinguished by the cell types (Fig. 1). In the anterior and posterior regions goblet cells are almost as numerous as columnar cells, the goblet cells have a fairly uniform diameter (Fig. 1*B*), and the striated border of the columnar cells is comparatively high. In the middle region each goblet cell is flask-shaped (Fig. 1*C*), it is separated from its neighbour by several columnar cells, and the striated border of the columnar cells is relatively low.

* Monsanto Chemicals Ltd. product.

The columnar cells have an appearance similar to those of the simple epithelial cells that occur in the midgut of many insects. The goblet cells have a basally placed nucleus surrounded by dense cytoplasm similar to that

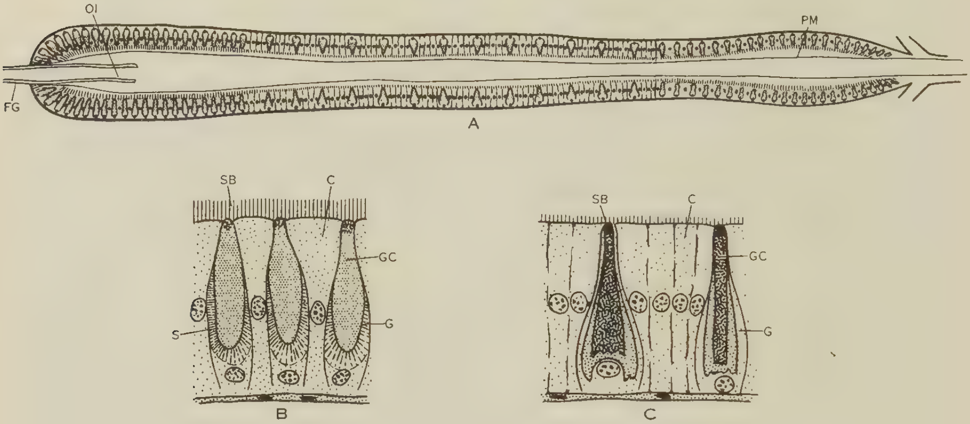

Fig. 1.—*A*, Midgut of a *Tineola* larva showing the distribution of columnar and goblet cells. *B*, The columnar and goblet cells of the anterior and posterior regions. *C*, The columnar and goblet cells of the middle region.
C, columnar cell; *FG*, foregut; *G*, goblet cell; *GC*, goblet cell cavity; *OI*, oesophageal invagination; *PM*, peritrophic membrane; *S*, striated lining to goblet cell cavity; *SB*, striated border.

of the columnar cells. The distal three-quarters of the goblet cell encloses a centrally placed cavity with a narrow, faintly striated lining. The tips of the goblet cells usually terminate about level with the striated border of the columnar cells and these cells, therefore, expose relatively little surface to the gut lumen in comparison with the columnar cells. The cavities of the goblet cells do not appear to open into the lumen, although the possibility of a narrow opening cannot be entirely discounted.

(*b*) *The Fate of Ingested Elements Capable of Forming Insoluble Sulphides*

Observations on larvae fed on diets enriched with salts of 19 elements are summarized in Table 1. It can be seen that both the food in the midgut and the faeces have a colour typical of the sulphides of the elements concerned and that the epithelium of both anterior and posterior regions of the midgut generally contains similarly coloured accumulations. Five of the elements resulted in sparsely scattered accumulations in the middle region of the midgut and one (tellurium) in heavy accumulations in this region. At times several additional metals (e.g. nickel) also resulted in barely detectable accumulations in this region. The colour of the faeces was sometimes influenced by the large amount (some 30-40 per cent.) of uric acid present in the pellets, which tended to give pellets a lighter shade than typical for the respective sulphide. Fuller data follow on representative metals to indicate the type of detailed results obtained. Except for the higher concentrations of arsenic, and to a

TABLE 1

COLOURS PRODUCED BY FEEDING *TINEOLA* LARVAE ON FABRICS ENRICHED WITH VARIOUS ELEMENTS
Blanks indicate colour similar to control. Asterisks indicate metals strongly accumulated by the goblet cells

Element	Formula and Colour of Sulphide	Colour of Faeces	Colour of Accumulations in Midgut: Anterior	Mid	Posterior	Colour of Food Undergoing Digestion	Salt Used
Zinc *	ZnS white		White		White	Whiter than usual	$ZnSO_4$
Iron	FeS black	Normal, light brown, or grey	Very light brown or few black masses (often no colour)		Light brown, sometimes black (often no colour)	Black, fluid sometimes blue-green	$FeCl_3$
Cadmium	CdS yellow-orange	Light yellow	Very light yellow			Light yellow	$CdCl_2$
Thallium	Tl_2S, Tl_2S_3 black	Grey	Black			Brown to black	$Tl(CH_3COO)$
Cobalt	Co_2S_3, CoS_2 black; CoS brown	Brown to black	Light brown		Very light brown	Dark brown	$CoCl_2$
Nickel *	NiS black	Dark brown or black	Black		Black	Dark brown	NiS $NiSO_4$
Tin	SnS_2 straw yellow	Greenish yellow	Very pale yellow	White specks	Very pale yellow		$SnCl_4$
Lead *	PbS black	Dark brown to black	Black		Brown to black	Dark brown to black	$Pb(CH_3COO)_2$
Antimony *	Sb_2S_5 orange	Greenish yellow	Orange	Orange specks	Orange	Light orange yellow	$SbCl_3$ Sb_2S_5
Bismuth	Bi_2S_3 brown	Brown or black	Brown to black		Brown to black	Dark brown	$Bi(NO_3)_3$
Arsenic	As_2S_3, As_2S_5 red or yellow	Light yellow to brownish yellow	Very light yellowish brown (sometimes)		Very light yellowish brown (sometimes)	Light brown (sometimes)	Na_3AsO_3
Copper	CuS, Cu_2S black	Brown	Light brown		Light brown to black	Dark brown	$CuSO_4$
Tellurium *	TeS_2 black	Black	Black	Numerous black masses	Black	Grey	Na_2TeO_3 Na_2TeO_4
Osmium *	OsS_2, OsS_4 black	Grey to black	Grey to black		Grey to black	Dark brown to black	OsO_4
Mercury *	HgS, Hg_2S black	Grey to black	Black	Black specks	Black	Black	$Hg(CH_3COO)$ $Hg(CH_3COO)_2$
Silver	Ag_2S black	Black			Few scattered black	Brown to black	Ag albuminate, $AgNO_3$
Palladium	PdS, PdS_2 brown to black	Dark brown	Brown			Dark brown	$Pd Cl_2$
Platinum *	PtS_2 black-brown	Light brown	Reddish brown	Very light brown	Reddish brown	Dark brown	$PtCl_4$
Gold *	Au_2S_3, Au_2S brown to black	Golden brown to brown	Golden brown to brown	Golden brown specks	Golden brown to brown	Golden brown	$HAuCl_4$

lesser extent thallium and tin, none of the elements appeared to have any adverse effects on the larvae.

(i) *Iron.*—The light brown, metal-linked fabric containing iron was seen to change in colour as it passed along the alimentary canal. In the foregut and the anterior fifth of the midgut the food was brown; it then changed to a dark green or black, which later gave way to a dark grey in the posterior end of the midgut. It appears that digestion of the fabric first results in visible liberation of iron about one-fifth of the distance down the midgut.

When fed on fabric dipped in 4 per cent. ferric chloride the epithelium of the anterior and posterior midgut regions had a light rusty-brown colour; with iron saccharate there were sometimes occasional black masses in the extreme anterior end and in the posterior fifth of the midgut.

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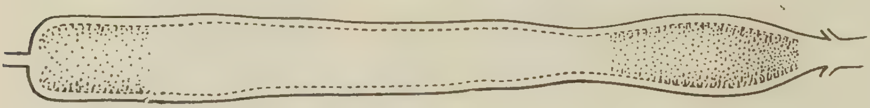

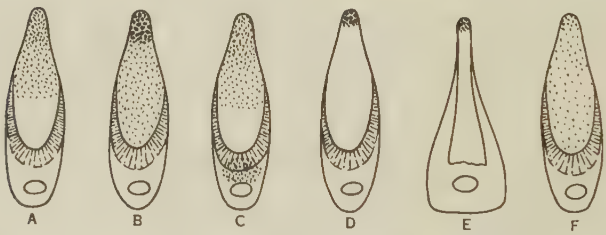

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Fig. 2.—Distribution of ferric iron in the midgut of *Tineola* larvae fed on iron-enriched wool. Iron accumulations indicated by stippling.

Fig. 3.—The site of metal accumulations in the goblet cells of *Tineola*. *A*, iron; *B*, nickel; *C*, copper; *D*, *E*, mercury; *F*, barium.

After staining for ferric iron by means of the Prussian blue reaction (potassium ferrocyanide and dilute hydrochloric acid) the anterior and posterior regions of the midgut stained heavily (Fig. 2). There was no staining of the remaining tissues, except very occasionally of the fat body or of the intima lining the hindgut. Sections of the midgut revealed that staining occurred only of the contents of the cavities of the goblet cells and then principally in the distal portion of the goblet (Fig. 3*A*). The cytoplasm of these cells and of the adjacent columnar cells remained unstained. Staining was generally heaviest in the goblet cells of the posterior midgut zone, less heavy in those of the anterior zone, whereas the goblet cells of the middle region failed to stain, indicating that goblet cells do not all function similarly. Except for the cut ends of the fibres, which reacted rapidly, the metal-linked fabric stained slowly after immersion in the ferrocyanide solution. However, the incompletely digested pieces of wool and other material in the faeces

stained very rapidly. Control larvae occasionally stained lightly for ferric iron in the anterior and posterior regions of the midgut.

Two hours after larvae had commenced feeding on a piece of iron-enriched fabric, which had also been dyed with eosin, dyed fibres could be seen to have reached the posterior end of the midgut. When these larvae were tested for iron the anterior midgut always became stained, whereas the posterior midgut reacted less frequently and then very weakly. The more usual condition with the posterior midgut staining more heavily for ferric iron than the anterior midgut became established only after some hours feeding. When larvae were transferred from iron-enriched fabric to control fabric the intensity of staining of the goblet cells gradually diminished over several days. However, it seldom disappeared entirely until moulting occurred. During moulting the old columnar and goblet cells are cast off into the midgut (Lotmar 1941) and the latter carry with them their accumulated iron.

When iron-fed larvae were tested for ferrous iron (potassium ferricyanide and dilute hydrochloric acid) the goblet cells of the posterior midgut stained intensely. In some larvae, but not all, there was some staining of the goblet cells in the anterior midgut, whereas the rest of the midgut remained unstained.

The occurrence of ferrous iron principally in the posterior midgut suggests that the oxidation-reduction potential of the goblet cell contents of this region is lower than that of the anterior midgut and it is noteworthy that black deposits of ferrous sulphide were observed more frequently in the posterior than in the anterior midgut.

The metal-linked fabric stained only very lightly and very slowly for ferrous iron and a similar behaviour was observed for the undigested wool in the faeces. However, the exterior of the faeces stained deeply and rapidly.

(ii) *Nickel.*—In living larvae fed nickel-enriched diets the anterior and posterior zones of the midgut could be seen through the transparent larval cuticle to contain elongated black masses. The actual distribution of dark masses within these zones depended upon the larval food. For example, on the nickel-linked wool, the masses occurred throughout the entire anterior midgut and were almost never seen in the posterior midgut (Fig. 4*A*). The light-coloured fabric in the anterior portion of the midgut became slightly darkened when it reached the middle of the midgut and the faeces were normal in colour. When larvae were fed on the fabric that had been dipped in 2 per cent. nickel sulphate the masses were present throughout the anterior midgut and in the central region of the posterior midgut (Fig. 4*B*). Larvae feeding on fabric dipped in 20 per cent. nickel sulphate (resulting in a 25 per cent. increase in fabric weight) accumulated dense black deposits throughout the entire anterior and posterior midgut (Fig. 4*C*), but no deposits could be seen in the middle region. The food throughout the midgut (and any fluid present in the foregut) was brown and the faeces were black. However, when the faeces were kept in a humid environment they became a greenish grey, presumably owing to the oxidation of nickel sulphide to nickel sulphate. Larvae fed on the yeast-casein diet containing 4 per cent. nickel sulphate accu-

mulated their densest and darkest masses in the middle of the posterior midgut (Fig. 4*D*). The food in the midgut was brown and the faeces black when excreted. It is clear, therefore, that the artificial diet contains enough sulphur to enable sulphide formation to occur readily, and in fact 0.5-1.0 per cent. cystine is present.

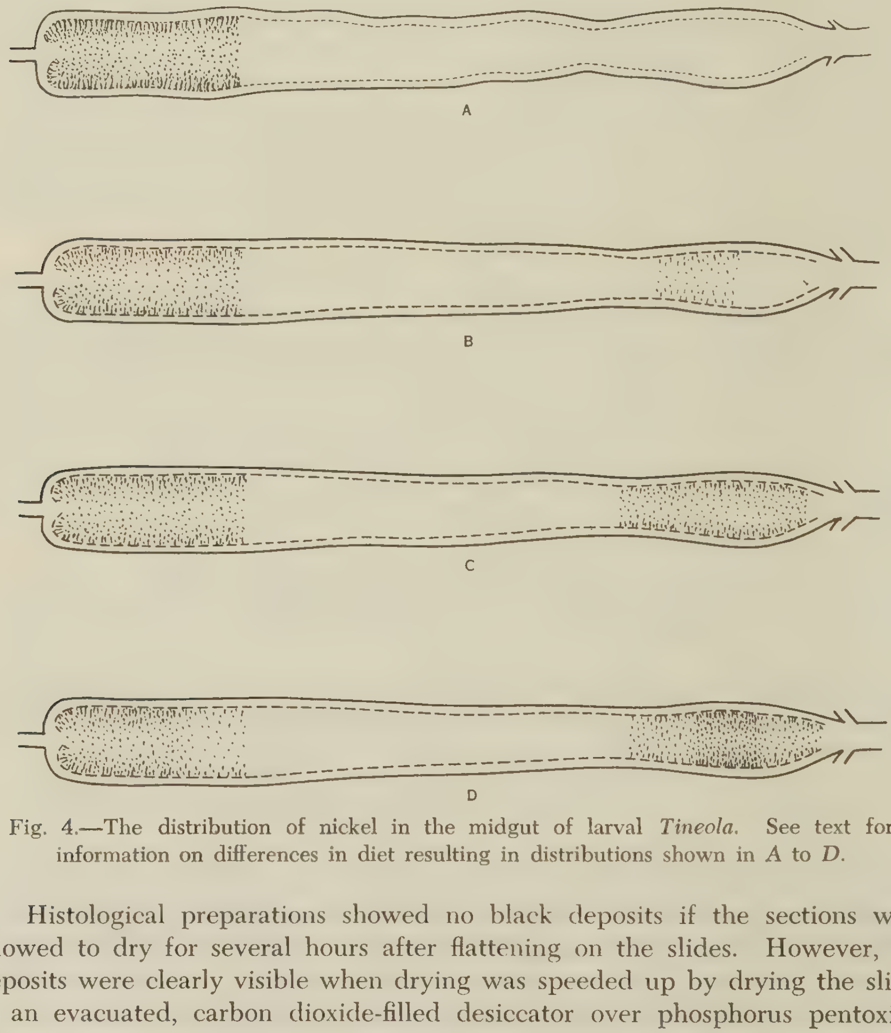


Fig. 4.—The distribution of nickel in the midgut of larval *Tineola*. See text for information on differences in diet resulting in distributions shown in *A* to *D*.

Histological preparations showed no black deposits if the sections were allowed to dry for several hours after flattening on the slides. However, the deposits were clearly visible when drying was speeded up by drying the slides in an evacuated, carbon dioxide-filled desiccator over phosphorus pentoxide. By this means oxidation of the moist deposits of nickel sulphide was prevented. Such histological preparations demonstrated that the black masses were the contents of the cavities of the goblet cells (Fig. 3*B*). The contents became progressively more heavily laden with black material towards the lumen of the gut and discrete black granules could be seen in the tips of many of the cells. The entire cavity appears to be filled after some days on a nickel diet whereas with some other metals (e.g. lead, Plate 1, Figs. 1 to 3) the deposits are restricted to the periphery of the cavity.

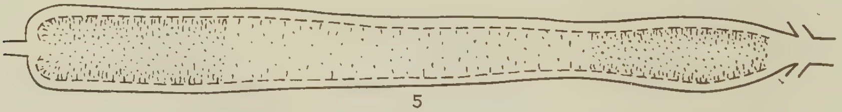
Following unsuccessful attempts with dimethyl glyoxime, salicylaldoxime, and α-furildioxime the presence of nickel was confirmed with a 0.1 per cent. solution of dithio-oxamide (rubeanic acid) in 70 per cent. alcohol. The deep purple nickel rubeanate formed proved fairly stable to normal treatment during sectioning and provided clear evidence that a nickel compound was distributed, as described above, in the cavities of the particular goblet cells concerned and nowhere else. Both uneaten fabric and faeces stained purple. No reaction was given by control larvae.

When larvae possessing conspicuous black goblet cell contents were transferred to control fabric the inclusions disappeared completely only during moulting. Prior to this there was some slight diminution of the amount of black material present. This appeared to be due, in part at least, to a sloughing off of entire goblet cells or to the discharge of the entire contents of their cavities since, in cleared whole preparations of the midgut, black cigar-shaped masses could occasionally be seen between the epithelial cells and the peritrophic membrane. It is also worth noting that a few goblet cells may be seen in sections to contain little or no black deposit, although the remainder are heavily laden.

(iii) *Copper.*—After feeding for several days on fabric dipped in 4 per cent. copper sulphate the anterior midgut was heavily laden with black masses (Plate 1, Fig. 4), whereas the posterior midgut contained fewer accumulations. The contents of the foregut and anterior midgut were light brown and those of the remainder of the midgut dark brown. When fed on lower concentrations of copper little or no accumulation could be seen, although the faeces were brown. However, when sections were prepared and mounted in sodium diethyldithiocarbamate in 20 per cent. alcohol, intense yellow staining of the anterior and posterior regions and faint staining of the middle region of the midgut were observed. This reaction was absent in control larvae. The most intense carbamate staining occurred either in the cytoplasm of the goblet cells immediately above the nucleus or in the distal two-thirds of the cavities of the goblet cells (Fig. 3*C*). The striated border throughout the midgut generally stained a light yellow and, in the anterior and posterior regions particularly, the lumen border of both goblet and columnar cells also stained. These observations indicate that a considerable amount of copper is present in these larvae in some form other than the free sulphide.

(iv) *Tellurium.*—Larvae that had fed on fabric treated with saturated solutions of sodium tellurate or tellurite accumulated black deposits in the goblet cell cavities in all regions of the midgut (Fig. 5). The faeces were black, although the fabric was white. These observations are of interest firstly because tellurium (a non-metal) is the only element observed to accumulate in quantity in the goblet cells in the middle region of the midgut, and secondly because they indicate that the phenomenon of the goblet cell accumulation (and colloidal dispersion with amino acids (see later)) is related to the sulphide nature of the compound rather than to its metallic nature.

(v) *Mercury.*—In larvae fed for long periods on fabric or on the artificial diet containing mercury (and they survived well on metal-linked fabric containing 25 per cent. by weight of mercuric acetate or cyanide) small black masses could be seen scattered throughout the epithelium of the anterior midgut (particularly at its anterior end) and of the anterior quarter of the posterior midgut (Fig. 6). There were also, quite often, small numbers of black masses present in the epithelium of the middle region and, on occasion, they were present only in this region. The black masses are jet black granules massed in the tips of some of the goblet cells (Plate 2, Figs. 1 and 2; Figs. 3*D* and *E*). In very occasional larvae, scattered black granules also occurred beneath the striated border of the columnar cells.



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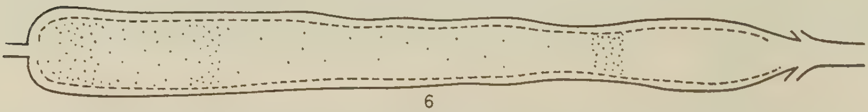

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Fig. 5.—The distribution of tellurium in the midgut of larval *Tineola*.
Fig. 6.—The distribution of mercury in the midgut of larval *Tineola*.

Following long immersion in a freshly prepared saturated solution of diphenyl carbazone in 70 per cent. alcohol, the characteristic purple colour produced with mercury could be seen around the edges of the black granules. The cavities of a few of the goblet cells also stained a purplish pink, and uneaten fabric and undigested wool in the faeces became purple. No reaction was given by control larvae or control fabric.

(vi) *Gold.*—Larvae fed on gold-enriched fabric accumulated conspicuous golden-brown masses in the goblet cell cavities in the anterior midgut and golden-brown to brown masses in the posterior midgut. The tips of the goblet cells in the middle region also frequently contained small golden-brown masses. Gold produced accumulations in these cells far more readily than any of the other metals tested.

(vii) *Elements Producing No Midgut Accumulations.*—Fabrics containing a number of other elements were also fed to *Tineola* larvae (Table 2), but no coloured compounds were accumulated in the midgut epithelium. However, in most instances this can be explained on the basis of the properties of the respective sulphides. Thus aluminium, cerium, chromium, and zirconium do not form sulphides in the presence of water and there is some doubt whether molybdenum trisulphide and uranyl sulphide would be precipitated under the alkaline conditions in the gut. Tungsten tends to form soluble thiotungstates under alkaline conditions, the trisulphide only being thrown down if the solution is subsequently acidified.

Selenates are reduced to selenites by H_2S and these produce a precipitate

consisting of selenium and sulphur, selenious sulphide being too unstable to exist under these conditions. It is possible, therefore, that the yellow coloration of food in the gut and of faeces is due to the presence of free sulphur.

Cerium and uranium caused the production of some faeces having a colour approaching that of the respective sulphides, but the remainder of the impregnated fabrics caused the production of normal coloured faeces. Although some of the treated fabrics were evidently toxic, some larvae survived on every fabric for a week or more, indicating that none of the compounds used was highly poisonous.

TABLE 2

COLOURS PRODUCED BY FEEDING *TINEOLA* LARVAE ON FABRIC ENRICHED WITH VARIOUS ELEMENTS

Blanks indicate colours similar to control larvae

Element	Sulphide	Faeces	Food in Gut	Salt Used
Aluminium	Is rapidly hydrolysed			$Al_2(SO_4)_3$
Cerium	Is rapidly hydrolysed	Pink to red		$CeCl_3$
Chromium	Is rapidly hydrolysed			$KCrO_4$
				$K_2Cr_2O_7$
Manganese	MnS green or pale pink			$MnSO_4$
				$KMnO_4$
Molybdenum	MoS_3 dark brown			$(NH_4)_2MoO_4$
Selenium	SeS_2 red-yellow	Yellow	Often light yellow	Na_2SeO_3
				Na_2SeO_4
Tungsten	WS_3 chocolate-brown			Na_2WO_4
Uranium	UO_2S brown-black	Grey		$UO_2(CH_3COO)_2$
Zirconium	Is rapidly hydrolysed			$Zr(NO_3)_4$

An unexpected inclusion in this group of elements is manganese, which does not result in sulphide formation, although it is closely related chemically to zinc, which does. A possible explanation is that the hydroxide is formed and this is not converted to the sulphide at pH 10. It may be significant also that manganese sulphide is far more soluble than zinc sulphide.

(*c*) *The Identity of the Coloured Compounds Accumulated in the Goblet Cells*

It is far too striking to be purely coincidental that not only the metal accumulations in the goblet cells but also the food in the midgut and the faeces invariably have a colour typical of the respective metal sulphides where these are insoluble. Furthermore, a number of elements that do not form insoluble sulphides under the conditions existing in *Tineola* larvae fail to produce visible accumulations when fed in the diet. Solubility experiments, such as those shown in Table 3, also indicate that the visible metal accumulations react as would be expected of finely divided sulphides.

It is of course well known (see Albert 1950) that metals form chelation compounds not only with amino acids but also with many other compounds occurring in tissues. There would be every opportunity for complex formation

to occur when amino acids are liberated from wool in the course of digestion in the alimentary canal or at a later stage when the metals are taken up by the midgut epithelium, where amino acids occur. However, these complexes, those of metals with polypeptides, pteridines, purines, porphyrins, and riboflavine are seldom black so that, even if they are formed, they cannot be the compounds (so frequently black) with which we are concerned.

TABLE 3

EFFECT OF IMMERSING METAL COMPOUNDS ACCUMULATED BY *TINEOLA* LARVAE IN VARIOUS SOLVENTS

U = unchanged, F = faded, — = decolorized

Solvent	Ni			Pb		
	15 min.	7 hr.	48 hr.	15 min.	7 hr.	48 hr.
Water	U	U	U	U	U	U
Ethanol	U	U	U	U	U	U
Xylene	U	U	U	U	U	U
1N HCl	U	F	—	U	—	—
0.1N HCl	U	U	—	U	F	—
1N HCl + $HClO_4$	F	—	—	U	—	—
1N HNO_3	U	F	—	F	—	—

(*d*) *The Source of the Sulphide Sulphur*

As outlined earlier, the highly reducing alkaline conditions encountered by wool in the larval midgut reduce the disulphide bond of cystine (which is present to the extent of about 13 per cent. by weight in wool) with the production of sulphydryl groups. The presence of these groups can, in fact, be readily demonstrated in the midgut lumen by the nitroprusside reaction. The reduced cystine is probably hydrolysed to give H_2S. When digestion occurs in the presence of metals it would be expected, therefore, that metal sulphides would be formed and there is no reason to doubt that this is the reaction leading to the production of characteristic colours in the alimentary tract following ingestion of metal-impregnated fabric.

Confirmation of this mechanism comes from an examination of the excreta of metal-fed larvae and from feeding larvae on metal-impregnated silk, which contains no sulphur amino acids.

Analyses (Powning, unpublished data) of faeces of larvae fed on control fabric demonstrate that they contain a good deal of cystine (6.65 per cent. of dry weight), presumably because this amino acid is present in such great quantities in wool that the larvae can utilize only a fraction of it for protein metabolism in conjunction with the amounts of other amino acids also available. When nickel was present in the diet (a fabric dipped in 20 per cent. $NiSO_4$, resulting in a 25 per cent. increase in weight) the cystine content of the faeces was very much lower (1.44 per cent. of dry weight). This indicates that nickel has combined with, and removed, some of the hitherto unutilized

sulphur. It becomes easier now to see why the presence of many metals in the larval diet has no detrimental effect.

Tineola larvae are unable to develop on silk, although they ingest it and remain alive, or even eventually pupate and produce adults, if transferred to it from a more adequate diet. When larvae were transferred from the standard woollen fabric to silk that had been dipped in 5 per cent. nickel sulphate there was some darkening of the food in the midgut, but no visible accumulation of nickel sulphide in the goblet cells. Since silk contains almost no sulphur the darkening is presumably due to the presence of sulphydryl groups carried over by the larvae from their earlier diet, and the presence of sulphydryl groups was, in fact, demonstrable by the nitroprusside reaction. The absence of goblet cell accumulations may be due either to the small amount of metal sulphide in the midgut or to the absence or inadequate concentration of suitable solubilizing agents (see later). The former was shown to be the correct explanation by the following experiments:

(i) When nickel sulphide is added to silk or "Terylene"* the sulphide accumulates in the goblet cell cavities. The faeces of silk-fed larvae are largely composed of undigested silk, indicating that little, if any, digestion has occurred. "Terylene" is a synthetic fabric and is completely resistant to digestion. It follows that the midgut is able to provide suitable conditions for the absorption of sulphides even if protein digestion is not taking place.

(ii) When 1 per cent. cysteine hydrochloride or 3 per cent. methionine or glutathione was added to a nickel sulphate-silk diet the food in the gut was considerably darker than without these sources of sulphur, the faeces were dark, and accumulations of nickel sulphide were almost always visible in the goblet cells of the anterior midgut, but not elsewhere. The formation of sulphide from methionine indicates that *Tineola* larvae are capable of demethylating this compound, thereby exposing the sulphur for sulphide formation. Demethylation is not perhaps surprising in view of the importance and frequent occurrence of enzymic transmethylation, at least in higher animals. Since the methionine content of wool is low (about 0.7 per cent.) this mechanism is probably not of great importance in sulphide formation from metals added to woollen fabrics.

From these various lines of evidence it is concluded that:

(i) The coloured compounds formed during digestion of metal-impregnated wool are sulphides;

(ii) The sulphur comes predominantly from sulphydryl groups produced by the reduction of cystine in the larval midgut;

(iii) There is a very considerable amount of unutilized cystine available for sulphide formation, and

(iv) Ingested sulphides can be absorbed by the midgut epithelium and accumulated in the goblet cell cavities.

* An ethylene terphthalate, a Dupont product.

(*e*) *Mode of Absorption of "Insoluble" Sulphides*

The accumulation in the goblet cell cavities of material having the same colour as that of the partly digested, element-enriched food would at first sight suggest that the sulphides are either produced *in situ* in the cavities, or that the coloured compounds are absorbed in solution from the gut lumen, and hence this could scarcely be as the insoluble sulphides. If the goblet cell cavity opened directly into the lumen it is, of course, possible that insoluble compounds could be taken up, but no opening can be seen in sections and there is also indirect evidence against such an opening. We have seen earlier that the compounds (e.g. those of iron and nickel) persist in the cavities for several days at least after nickel-fed larvae have been transferred to control fabric. This is evidence either that there is no discharge from the cavities into the gut lumen (and discharge is not yet proven, although it is generally accepted) or that discharge occurs either through a cell membrane or through a very narrow opening, which holds back much of the accumulated metal. If there is an opening through which the sulphide has been taken up it should be large enough to permit its discharge later. If, alternatively, there is a bounding cell membrane difficulties arise, as mentioned above, in providing an explanation for the uptake of insoluble compounds through it.

The capacity of the midgut epithelium to absorb and accumulate sulphides was clearly demonstrated by feeding larvae on woollen fabrics or silk smeared with the freshly precipitated, thoroughly washed, sulphides of several of the metals. A curious observation was that when ferrous sulphide was ingested a bluish green fluid could sometimes be seen between the peritrophic membrane and the epithelial cells, although black accumulations in the goblet cell cavities were not observed.

It appears that the mechanism by which the sulphides are rendered capable of being absorbed is that described by Neuberg and Mandl (1948). These authors showed that metal sulphides are "solubilized" by the presence of amino acids and polypeptides, and that, although the complexes produced are often colourless, some of those formed with iron sulphide are bluish green. They concluded that the sulphides formed true solutions with amino acids principally because of (i) their stability, in some cases for several days, (ii) their clarity, (iii) their lack of a Tyndall effect at ordinary observation, and (iv) the absence of flocculation with NH_4OH–NH_4Cl or NH_4OH–$(NH_4)_2SO_4$. However, a re-examination of the problem (Harris, unpublished data) has shown that the "solutions" are colloidal since the metal sulphides can be readily flocculated by the addition of large amounts of $NaNO_3$ solution and much smaller amounts of $CaCl_2$ or alum. Furthermore, in the copper "solution" negatively charged colloidal particles were observed to migrate under the influence of a current.

Amino acids and polypeptides are liberated from the food during larval digestion or are secreted with the digestive juices and it is suggested that these then produce an extremely finely divided colloidal dispersion of portion

of the metal sulphide also produced. Any undispersed sulphide is excreted. These processes can apparently be observed sometimes for ferrous sulphide, for bluish green fluid occurs between the peritrophic membrane and the epithelial cells. However, until more is known of the environment in which the sulphides are deposited in the cells and of the polypeptides and amino acids liberated from wool during its digestion by *Tineola* larvae, it will not be possible to explain the few minor irregularities observed in sulphide accumulation in the goblet cells.

Since the columnar cells expose a very much greater surface area to the gut lumen than the goblet cells it would be expected that most of the products of digestion would be absorbed by them. It is suggested, therefore, that the colloidal sulphides may first be taken up by the columnar cells and passed without accumulation into the goblet cells, which in turn discharge them into their cavities. A difficulty arises, however, in explaining on this basis the path of uptake in the central region of the midgut. Here, unlike the anterior and posterior regions, most of the columnar cells are not in contact with goblet cells (Fig. 1) and would, therefore, have greater difficulty in disposing of absorbed sulphides. It should be pointed out, however, that the goblet cells of this region accumulate sulphides and dyes (see later) less readily than the goblet cells of other regions and this may be correlated with a different absorptive capacity of the columnar cells.

The generally accepted function of goblet cells is that they accumulate secretions (notably digestive enzymes) in their cavities prior to discharge into the gut lumen. If this is so, the enzymes, salts, or other materials encountered in the cavities may well act as flocculating agents. Alternatively, if the dispersion-producing amino acids are reabsorbed the colloidal dispersion will become unstable and sulphide will be deposited in the cavity. If the secretions of the cavities are discharged continuously through a bounding membrane into the gut lumen the liberated particles of sulphide would be carried by the flow of secretion into the necks of the cavities and would first accumulate here and only later in the remainder of the cavity. This is what appears to happen.

(*f*) *The Fate of Ingested Metals Capable of Forming Insoluble Phosphates*

A mechanism has been demonstrated whereby *Tineola* larvae can detoxify elements that form insoluble sulphides. There are, however, other metals, such as barium and beryllium, that are toxic to many animals but form readily hydrolysable sulphides. It was therefore of interest to determine the effect of feeding these metals and also of feeding calcium and magnesium which have certain properties in common with barium and beryllium, notably that they all form relatively insoluble phosphates.

(i) *Barium*.—The presence of barium in granules that occur near the lumen border of the columnar cells in the anterior and posterior midgut of larvae feeding on a normal diet has already been demonstrated by the rhodizonate technique (see Waterhouse 1951, Fig. 2). After feeding on barium-

enriched wool these two regions of the midgut stain more heavily than usual, but the middle region, as before, remains unstained. In both anterior and posterior regions, the distribution of staining in the cells was similar (Plate 2, Fig. 3). Stained granules occurred in the inner ends of the columnar cells and, at the same level but far less frequently, in the goblet cells (Fig. 3*E*). In the columnar cells these granules are often separated from the striated border by a narrow zone of granule-free cytoplasm although, at times, the granules do occur directly beneath the striated border. The structureless contents of the cavities of the goblet cells may stain a uniform pink, particularly at the extreme anterior and posterior ends of the midgut. The nuclei of the goblet, columnar, and regenerative cells also stain a diffuse light pink with rhodizonate but the nucleoli are the only really conspicuously staining elements. At times the regenerative cells throughout the midgut were found to contain large numbers of granules which stained intensely for barium (see Waterhouse 1951, Plate 1, Fig. 2).

(ii) *Beryllium.*—Larvae were fed with little mortality for a week or more on fabrics dipped in various concentrations (up to 10 per cent.) of beryllium sulphate or nitrate. The histochemical distribution of beryllium in the midgut epithelium was examined by means of the napthochrome green *B* technique (Denz 1949). On low beryllium concentrations the apple-green staining characteristic for beryllium was localized in the lumen border of the columnar and goblet cells in the region of the accumulation of the barium-rich granules. Light staining also occurred in the middle region of the midgut. With higher concentrations of beryllium the entire midgut epithelium stained a light green. It does not appear, therefore, that the resistance of larvae to beryllium poisoning is to any appreciable extent dependent upon its detoxification as insoluble phosphate, although some is undoubtedly deposited in this form. The low toxicity of beryllium to *Tineola* larvae is interesting in view of its high toxicity to mammals, where it has been shown to inhibit alkaline phosphatase and other magnesium-activated enzymes (Aldridge 1950). The present experiments suggest that alkaline phosphatase is not an important enzyme in the *Tineola* midgut, which is in agreement with the report that it could be detected only faintly in some of the columnar cells (Day 1949*b*).

(iii) *Calcium.*—Sections of *Tineola* midgut were stained by the Gallamine blue technique for calcium (Stock 1949). This indicated the presence of calcium in the granules, which occur in the anterior and posterior midgut in the columnar cells and less frequently in the tips of the goblet cells. Occasionally granules in the regenerative cells also stain for calcium. This finding confirms the report of Lotmar (1941) who used Kossa's method. The calcium is present in the granules which have been shown above to contain barium. They do not occur in all larvae feeding on fabric, although they are numerous in larvae on a calcium-enriched diet.

(iv) *Magnesium.*—Treatment of fixed control larvae with 0.2 per cent. Titan yellow, followed by the addition of a drop of 10 per cent. sodium

hydroxide produced a red stain in the midgut, the cut edges of the cuticle, and occasionally in the salivary glands. This is not surprising in view of the fact that magnesium is a normal tissue constituent. More intense staining occurred in larvae fed on a magnesium-enriched fabric. Staining was generally most intense in the anterior quarter of the posterior midgut, less intense in the remainder of the posterior midgut and in the anterior midgut, and comparatively light in the mid midgut. Similar results were obtained with quinalizarin and less satisfactory ones with *p*-nitrobenzene azo-α-naphthol (Magneson).

Permanent sections could not be made of material stained with Titan yellow since the red stain faded very rapidly in xylene to a pale yellow. However, if fixed tissues were sectioned and mounted in alkaline Titan yellow, the inorganic granules shown earlier to be the site of barium and calcium accumulations could be seen to have taken on a pink or red coloration. Quinalizarin-stained material enabled permanent stained preparations to be obtained.

The stained granules occurred most frequently near the lumen border of the columnar cells in the regions described above and were occasionally seen in the tips of the goblet cells. At times too, the cavities of the goblet cells were lightly stained. When the regenerative cells along the entire midgut commenced to enlarge in preparation for the next moult they frequently contained numerous granules that stained for magnesium.

(v) *The Presence of Insoluble Phosphates.*—The cobalt sulphide method for demonstrating phosphates (Danielli 1946) indicated that the granules, which have been shown under appropriate conditions to be sites of barium, beryllium, calcium, and magnesium deposition, also contain phosphate. Granules extremely rich in phosphate were particularly numerous in the inner ends of columnar cells of larvae that had fed on the yeast-casein diet enriched with calcium glycerophosphate (Fig. 7). A few granules occurred in the tips of some of the goblet cells and phosphate was also, at times, detected in diffuse form in the cavities of these cells. It is highly probable, therefore, that any slight excess in the diet of the above metals (and of any others capable of forming insoluble phosphates) will be immobilized as phosphates in the midgut epithelium. The addition of dilute acid to the midgut did not produce any visible evolution of gas, indicating that little if any carbonate can be present in the granules. It appears that the granules are not dissimilar in composition to those accumulated in the larval malpighian tubules of the blowfly *Lucilia cuprina* (Waterhouse 1950).

(g) *The Fate of Fluoride*

When larvae were placed on fabric that had been dipped in 1 or 4 per cent. sodium fluoride fairly high mortality occurred, although a number were still alive after one week. The presence of odd fibres in the midgut was evidence that these living larvae had ingested some of the fabric, although the

relatively few faeces produced indicated that it was distasteful to them. It appears, therefore, that the larvae are able to dispose of a certain limited amount of ingested fluoride. Since calcium is often present in sufficient amount to permit the formation of calcium-rich granules in the midgut it is suggested that absorbed fluoride may be prevented from exerting toxic effects by rapid deposition as calcium fluoride in the granules. The situation of the granules near the lumen border of the cells would enable this deposition to occur without the necessity of transporting the fluoride far into the cell interior. However, since the amount of calcium present in the fabric must be extremely small, many larvae evidently soon reach a stage at which all ingested fluoride cannot be detoxified.

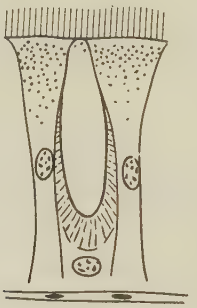

Fig. 7.—The distribution of phosphate granules (indicated by stippling) in the columnar cells of the anterior and posterior midgut.

(*h*) *Effect of Feeding Boric Acid and Borax*

Larvae developed satisfactorily and appeared quite normal on fabrics that had been dipped in warmed 10 per cent. solutions of boric acid or borax. This is interesting in view of the toxicity of these compounds to larvae of *L. cuprina*, which also develop on a diet rich in animal protein (Lennox 1941). However, the most important proteolytic enzyme in blowfly larvae appears to be adenosine deaminase which is inhibited by boric acid, and which must play little, if any, part in the digestion of wool by *Tineola* larvae.

(*i*) *Selective Accumulation of Dyes by the* Tineola *Midgut*

The selective accumulation of needle-shaped crystals in the columnar cells of the middle region only of the *Tineola* midgut after feeding on a dyed fabric has been recorded (Day 1951). It was therefore of interest to determine whether other dyes were similarly treated, for this would indicate a highly specific function for these particular cells.

A careful re-examination of the distribution of dye accumulated by larvae feeding on the same dyed fabric as used by Day demonstrated that the dye was invariably restricted to the goblet cell cavities and did not occur in the columnar cells. It was accumulated in a narrow band of goblet cells at the

end of the anterior midgut and also in the tips of the goblet cells of the middle region in the same location as the sulphides mentioned earlier.

Fabrics dipped in 1 per cent. ninhydrin, Trypan blue, or 12 vital dyes were also fed to *Tineola* larvae (Table 4). Ninhydrin and eight of the dyes were accumulated more or less strongly in the anterior midgut, and accumulations of many of these dyes could be seen in the goblet cells. The precise location of most dyes was not determined. However, methylene blue was

Table 4

THE DISTRIBUTION OF DYES IN THE MIDGUT OF *TINEOLA* LARVAE FED ON DYED FABRIC

+++ Very heavy; ++ distinct; + less heavy; ± just distinguishable accumulations; blank, no accumulations

Dye	Midgut: Anterior	Midgut: Middle	Midgut: Posterior	Remarks
Benzopurpurin 4B				
Brilliant cresyl blue				
Brilliant vital red				
Chicago blue	+ goblet cells		±	
Dianyl blue	+ goblet cells			
Janus green	+ goblet cells			
Methyl violet	++ in few goblet cells	+++		
Methylene blue	++	+		Fabric blue in ant. end ant. midgut, elsewhere colourless
Neutral red	+++ all cells?			
Ninhydrin	++ pink mainly in goblet cells		±	Fabric blue in ant. and post. midgut, white in mid midgut. Faeces pink
Pontamine sky blue	+		±	
Trypan blue	+			
Vital new red				
Vital red				

rendered insoluble by the ammonium molybdate procedure and its distribution examined in sections. Fully coloured methylene blue occurred mainly in the cytoplasm of the anterior goblet cells and the lining of the cavities of the goblet cells of the middle region. When the epithelium was heavily laden with dye, small, scattered granules of methylene blue also occurred near the basement membrane and throughout the columnar cells. It is clear that these regions cannot be as intensely reducing as the midgut digestive juices. Methyl violet appeared in living larvae to have a similar distribution. Only three of the dyes were visibly accumulated by the posterior midgut epithelium. It is clear, therefore, that the three regions of the midgut accumulate dyes very differently. These experiments with dyes provide further evidence that the cells of the various regions of the midgut are capable of acting in an independent and highly specific manner.

(j) *Fate of Ingested Metals in Other Lepidopterous Larvae*

The structure of the goblet cells and midgut epithelium of the species mentioned below will be described elsewhere (Waterhouse 1952*a*).

Larvae of *Plutella maculipennis* (Curtis) (the diamond-back cabbage moth) were reared on seedlings of Chihili (the dwarf Chinese cabbage) whose roots were immersed in 0.5 per cent. solutions of ferric chloride or copper sulphate. When larvae that had fed on the iron-enriched diet were tested, an intense Prussian blue reaction was given by most of the goblet cells (Fig. 8*A*), but not by any of the columnar cells. Staining was confined to the cytoplasm. It was strongest between the nucleus and the striated lining of the cavity and often extended up around this lining towards the gut lumen. Iron occurred less frequently between the nucleus and the base of the cell. The nucleus, the striated lining, and the contents of the cavity were not observed to stain. This distribution of iron in the cytoplasm of the goblet cells is not inconsistent with the possibility that it has been transferred laterally after absorption by the columnar cells.

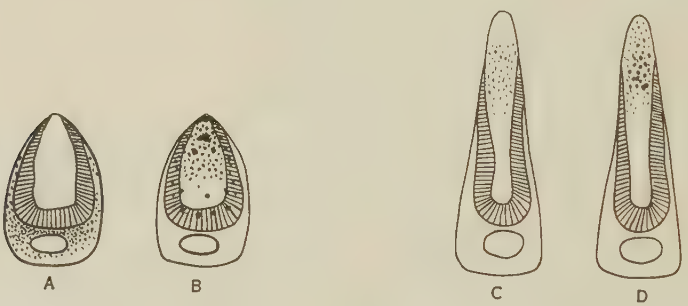

Fig. 8.—*A*, The distribution of iron in a goblet cell of *Plutella*.
B, Copper in a goblet cell of *Plutella*.
C, Iron in a goblet cell of *Heteronympha*.
D, Copper in a goblet cell of *Heteronympha*.

After copper feeding, the carbamate test demonstrated that this metal was present mainly in the goblet cells, although it could be detected in small amounts elsewhere in the midgut epithelium. In the goblet cells copper was restricted to the cavity and its lining, appearing as numerous scattered golden-brown masses in these situations (Fig. 8*B*). The tip of the cell often contained a large mass of copper-rich material.

Larvae of *Heteronympha merope* (Fabr.) (the "Common Brown" butterfly) were reared on a variety of native grasses, the roots of which had been immersed in metal solutions. Small, dark brown masses are often present throughout the midgut epithelium of control larvae. These masses, which are most abundant in the middle region, were shown by sectioning to be the contents of the goblet cell cavities. After iron feeding, small accumulations of ferric iron could sometimes be detected in the distal half of the cavity of some of the goblet cells (Fig. 8*C*). No iron could be seen in the columnar

cells. After copper feeding the distal half of some of the goblet cell cavities and the adjoining striated lining stained yellow with carbamate (Fig. 8*D*). Some copper-rich masses were present, but the distribution of staining was generally far more uniform than in *Plutella*. At times the striated borders of the columnar cells stained a light yellow, but the remainder of these cells did not react.

Iron accumulations were also detected in the goblet cells of three other species. As in *Tineola* and *Heteronympha*, it was present in the goblet cell cavities of *Pieris rapae* and *Galleria mellonella* but, like *Plutella*, it occurred in the cytoplasm of the goblet cells in *Ephestia kuhniella*. From these scattered observations it is clear that the goblet cells of many lepidopterous larvae accumulate iron and copper and presumably other metals also.

Barium-rich granules were detected in the midgut epithelium of several species fed on a barium-enriched diet. These granules occurred principally near the lumen border of the columnar cells and less frequently in the goblet cells.

IV. Discussion

The foregoing experiments demonstrate that the goblet cells in the midgut of *Tineola* and other lepidopterous larvae play a specialized and important part in the disposal of a large number of absorbed metals and one non-metal, tellurium. It is clear, however, that all goblet cells do not behave in an identical fashion. There are firstly species differences in the manner in which ferric iron is accumulated. In *Tineola, Heteronympha, Pieris,* and *Galleria* it is present in the cavity, whereas in *Plutella* and *Ephestia* it is in the cytoplasm. Secondly, in *Tineola larvae,* the goblet cells in the middle region of the midgut (in which relatively few metals were accumulated) function differently from those in the anterior and posterior midgut regions. This is perhaps not unexpected in view of the different morphological appearance of the goblet cells in these regions. Thirdly, and more remarkable, is the differential behaviour in *Tineola* of the goblet cells in the anterior and posterior zones to different metals. This cannot simply be a question of the availability of the sulphide. For example (Figs. 2, 4, 6) the goblet cells in the posterior midgut all accumulate iron at low concentrations in the diet, but only those at the anterior end of this region accumulate nickel unless the nickel concentration is high. Furthermore, only a few of those that accumulate nickel ordinarily accumulate mercury. This indicates that cells that have a similar histological appearance and behave similarly to one metal behave differentially to others. This differential behaviour is not confined, however, to the goblet cells, for the columnar cells in the anterior and posterior regions accumulate granules, which are not present in the columnar cells in the middle region. These granules enable the inactivation of a certain limited amount of absorbed alkaline earth metals and possibly also of fluoride. Except for small amounts of ascorbic acid (Day, unpublished data) no capacity for accumulation has yet been detected in the columnar cells of the middle region of the midgut, which may be concerned mainly with secretion. Differential accumulation is not restricted to cells of

the lepidopterous midgut, however, for iron and copper are accumulated only by certain midgut cells of *Lucilia* larvae (Waterhouse 1940, 1945) and *Drosophila* larvae (Poulson 1950*a*, 1950*b*).

Other interesting information on the distribution of materials in the midgut of *Tineola* larvae is also available. Thus the columnar cells throughout the midgut are reported to possess a weak alkaline phosphatase and to contain granules that give the silver reduction test for ascorbic acid (Day 1949*a*, 1949*b*). These latter granules occupy a position identical with those now shown to contain calcium, magnesium, and phosphate, and this raises some doubt of the validity of the positive test for ascorbic acid. The goblet cells, on the other hand, do not contain alkaline phosphatase, ascorbic acid, or mucoid material (Day 1949*a*, 1949*b*, 1949*c*).

There are considerable differences between metals in the amounts of sulphide accumulated in the goblet cell cavities. Elements marked with an asterisk in Table 1 accumulate readily, whereas others accumulate slowly or only in occasional larvae. This is doubtless related to the properties of the various sulphide-amino acid colloidal dispersions and to the conditions they encounter in the midgut epithelium. When more is known it may be possible to explain, for example, why neither copper nor iron regularly appear in the cavities as sulphides, although their presence there in some other form can be readily demonstrated by appropriate histochemical tests. It is probable that these elements are being absorbed, in part, by a more usual mechanism than solubilization of their sulphides. It is not known, for example, whether the colour of the sulphide is masked, owing to the fact that these metals are still dispersed by amino acids. This raises the possibility that there may be definite, but invisible, accumulations of metals elsewhere in the epithelium. If this were so, the metals must be present in some form that does not react with available histochemical tests, for such tests have demonstrated that heavy accumulations of iron, nickel, copper, lead, and mercury are largely restricted to the goblet cell cavities.

It has been stated that digestion of wool first commences at the beginning of the middle region of the midgut, since visible changes in the fibres are first detected at this level (Day 1951). However, reducing conditions, which are capable of promoting the rupture of the disulphide bonds, are established in the anterior region also, as can be demonstrated by feeding larvae on oxidation-reduction indicators (Waterhouse 1952*b*). The appearance of iron in the anterior goblet cells (but seldom in those of the posterior midgut) by the time iron-enriched fabric has travelled to the end of the midgut suggests that some digestion and absorption take place in the anterior midgut also.

The function of the metal accumulations in the goblet cells warrants consideration. If the metals have been taken up, as is possible, via the columnar cells, then their location in the portion of the goblet cells adjacent to the lumen would suggest that this may be principally a form of storage excretion of metal absorbed in excess over metabolic needs, or that the metals are being prepared for active elimination into the gut. For the latter to be efficient,

however, the metals would have to be eliminated from the anterior midgut in a form that would not permit re-absorption further down the gut. The elimination of metal accumulations when the entire old epithelium is discarded at moulting strongly supports the view that they constitute a form of storage excretion.

Some information on the mode of secretion of the goblet cells is available from the persistence of metals in their cavities for several days after metal-fed larvae have been transferred to control fabric. This is evidence that, if any regular passage of material from their cavities into the lumen occurs, it is either through a very narrow opening which holds back much of the accumulated metal, or through a cell membrane. If the entire contents of the cavity are discharged into the gut either regularly or at a particular stage in cell maturation the accumulated metals would inevitably be discharged at the same time. If periodic discharge does occur the present observations indicate that the frequency of such discharge cannot be greater than every two or three days. It has been mentioned earlier that, in sections, there appears to be a bounding membrane between the goblet cell cavity and the gut lumen. The functional evidence just presented supports this interpretation and is regarded as being more satisfactory than the histological evidence. Firstly, the latter is contrary to the observations of previous authors who have considered that the cavity opens directly into the lumen, as in the differently functioning goblet cells of mammals. Secondly, it is almost impossible when examining even thin sections to decide whether one is focusing on a bounding cell membrane or on the obliquely cut end of a channel leading from the goblet cell cavity into the gut lumen. However, in the many sections of several species of larvae examined no goblet cell cavity could be seen communicating directly with the lumen unless fixation of the tissues was clearly faulty. Thus, although there is not yet conclusive evidence that the goblet cell cavity is closed available evidence favours this view. Alternatively, if the sulphides are absorbed directly from the lumen by the goblet cells it will be necessary to recast current ideas of the function of the goblet cells, namely that they are principally secretory in nature.

The foregoing experiments demonstrate that *Tineola* larvae, partly by virtue of their unusual diet and partly because of their digestive mechanisms, are singularly well equipped to detoxify often quite large amounts of inorganic materials that are ordinarily regarded as highly toxic to animal life. Probably the most toxic of the relatively few elements employed in the present tests that had adverse affects on the larvae were fluorine, arsenic, and thallium, but the larvae tolerated relatively high concentrations even of these. However, accurate data on toxicity were not obtained. Unless the larvae possess a more effective mechanism than was discovered for the detoxification of fluorides, fluorine would appear to offer better possibilities for mothproofing than the other elements tested. It is perhaps significant that fluorides (mainly silicofluorides) are the only inorganic materials that have found any widespread use for mothproofing fabrics (Hartley, Elsworth, and Barrett 1943).

It is interesting to speculate on the possible importance in metal metabolism of the colloidal dispersion of insoluble sulphides. Sulphides are frequently formed in the digestive tract of animals, largely by bacterial action on organic sulphur compounds (Fromageot 1947). Under these conditions insoluble sulphides of many essential minerals are probably formed and these would be lost to the animal by excretion unless they are capable of being absorbed after colloidal dispersions have been formed. Since animals possess the capacity to oxidize sulphide to sulphate (Fromageot 1947) it is possible that the uptake of essential minerals as sulphides and their transport in the body in this form before utilization may be of fairly general occurrence. Metals absorbed as ions may also form sulphides or mercaptides after reaction with sulphydryl groups in the tissues, and transport of sulphides and the liberation of ions by subsequent oxidation to sulphate may play an important part in mineral metabolism.

It is also worth considering the general evolutionary significançe of the sulphide-detoxifying mechanism. This is reminiscent of the protection given by 2-3-dimercaptopropanol (British Anti-Lewisite) against poisoning by arsenic and a number of other metals. Clothes moth larvae possess an extremely effective biochemical mechanism for detoxifying many metals they would never normally encounter in their diet. Not only is the mechanism able to deal effectively with very large quantities of metals, but the range of insoluble sulphides formed is greatly extended by the fact that the conditions are quite alkaline. As Professor Dobzhansky has pointed out (in personal discussion) this is indeed a remarkable instance of biochemical "pre-adaptation" to a change which has not yet occurred, and may never occur, in their environment. It is possible that many other processes, which are now essential to the life of organisms, may have arisen initially as fortuitous by-products of some other metabolic process.

V. Acknowledgments

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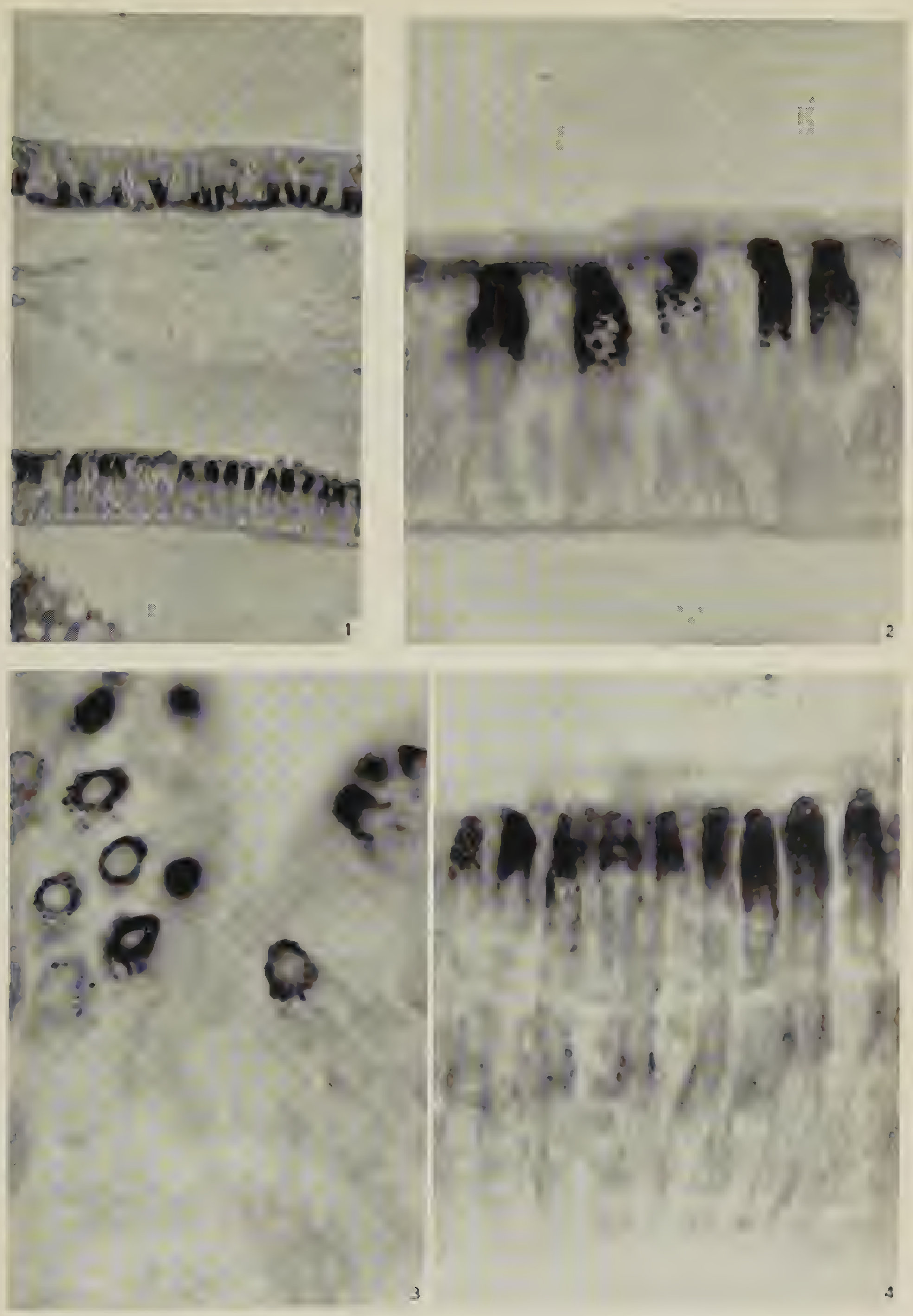

Fig. 1.—L.S anterior midgut of a *Tineola* larva, showing accumulations of lead sulphide in goblet cells.

Fig. 2.—As Figure 1, but at higher magnification.

Fig. 3.—Tangential section showing lead sulphide at periphery of goblet cell cavities.

Fig. 4.—L.S. anterior midgut of a *Tineola* larva, showing copper sulphide accumulations.

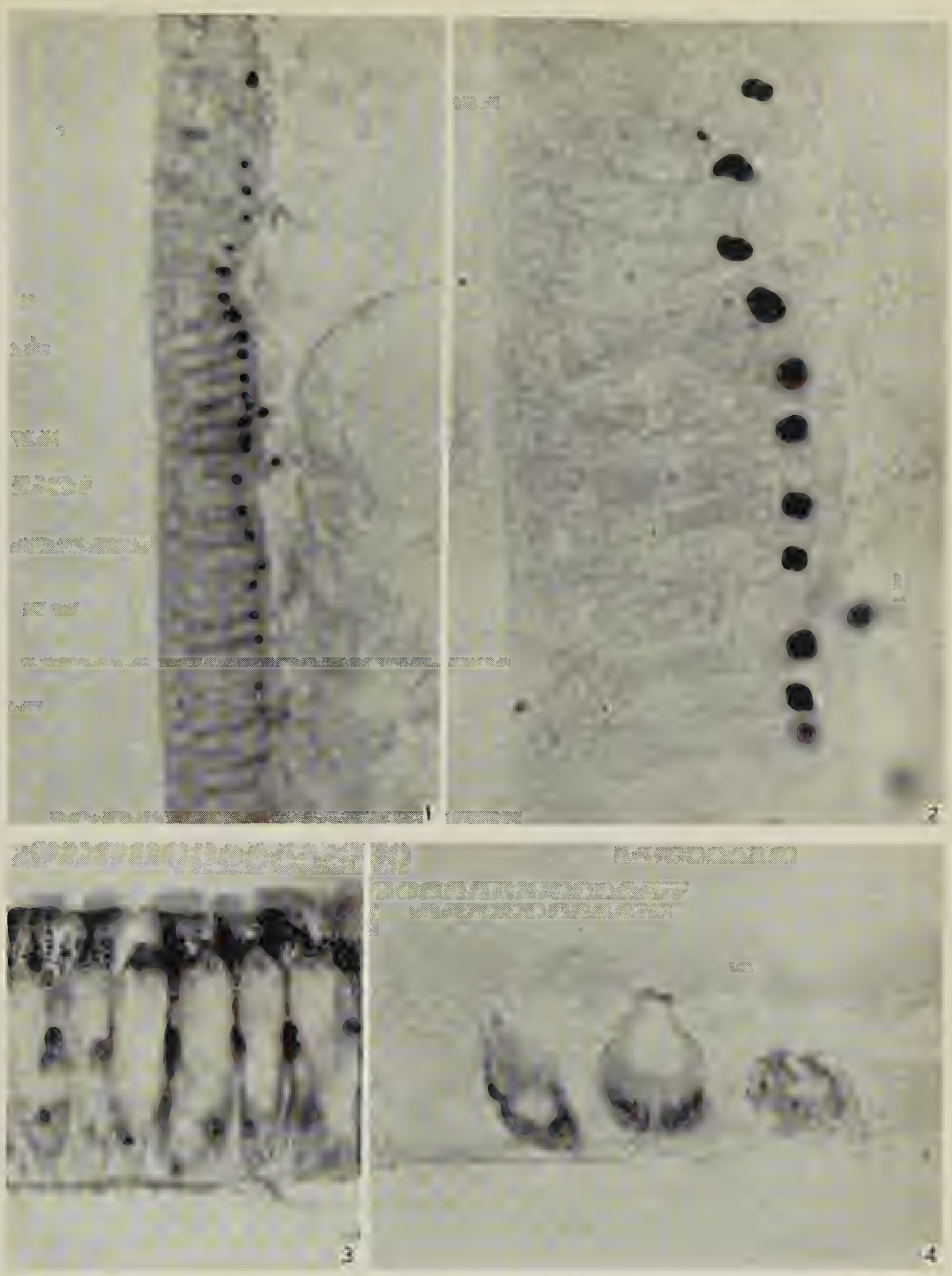

Fig. 1.—L.S. anterior midgut of a *Tineola* larva, showing accumulations of mercuric sulphide in tips of goblet cells.

Fig. 2.—As Figure 1, but at higher magnification.

Fig. 3.—L.S. beginning of anterior midgut of a *Tineola* larva, showing granules (stained for barium) near lumen border and principally in the columnar cells.

Fig. 4.—L.S. *Plutella* midgut, stained with the Prussian blue reaction, showing the accumulation of ferric iron in the cytoplasm at the base of the goblet cells.

STUDIES ON THE DIGESTION OF WOOL BY INSECTS

V. THE GOBLET CELLS IN THE MIDGUT OF LARVAE OF THE CLOTHES MOTH (*TINEOLA BISSELLIELAL* (HUMM.)) AND OTHER LEPIDOPTERA

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Summary

Goblet cells and columnar cells occur, together with regenerative cells, in the midgut epithelium of lepidopterous larvae. The columnar cells have an appearance typical of the simple epithelial cells that occur in the midgut of many insects. The goblet cells are highly differentiated and, although there are marked variations between species, such as in frequency of occurrence, in shape, in staining reactions, and so on, their basic structure is very similar. Bodian's 'Protargol' staining technique provides excellent differentiation of goblet cells. Each goblet cell has a basally situated nucleus and contains an internal cavity, which is bordered by a faintly striated lining. No opening permitting direct movement of material from the cavity into the lumen has been observed. Available evidence suggests that materials moving out of the cavity pass through a bounding membrane. Unlike columnar cells, goblet cells do not possess a striated border on their lumen surface.

There is no satisfactory evidence for the occurrence of goblet cells in orders other than Lepidoptera.

I. Introduction

The regular occurrence of two principal cell types (columnar cells and goblet cells) in the midgut epithelium of lepidopterous larvae has been known for many years. Much of the early literature on the structure of the goblet or caliciform cell and its relationship to the columnar cell has been reviewed by Buchmann (1928), Henson (1929), Lotmar (1941), and Woke (1941). It was once thought that goblet cells were produced from columnar cells by discharge of material, followed by an invagination of the striated border, but there is no satisfactory evidence to support this view. On the other hand, it is now known that goblet cells are present before the larva has hatched from the egg (Woke 1941). Furthermore, when the epithelium is cast off and renewed at each moult, goblet cells are differentiated directly from the regenerative cells and are visible, with their developing internal cavity, before the new epithelium has taken on any digestive function (Lotmar 1941).

Several authors (Day 1951; Lotmar 1941; Woke 1941) have suggested that the goblet cells are secretory and function principally in the production of digestive enzymes, which are stored in the goblet cavity prior to discharge

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into the lumen of the gut. As yet, there is no evidence to support this hypothesis, although there are some data that render it improbable (Waterhouse 1952*b*). In larvae of the clothes moth, *Tineola bisselliella*, the goblet cells do not contain mucoid material, ascorbic acid, or alkaline phosphatase, although ascorbic acid granules and a weak alkaline phosphatase have been reported in the columnar cells (Day 1949*a*, 1949*b*, 1949*c*). However, in *Tineola* and in several other lepidopterous larvae, goblet cells play an important part in metal and dye metabolism, high concentrations of metallic salts and dyes being accumulated in the goblet cavity or in the cytoplasm of the cell (depending upon the species and the material accumulated). On the other hand, metal accumulations cannot, with few exceptions, be detected in the columnar cells (Waterhouse 1952*a*, 1952*b*). In conjunction with these experiments on metal detoxification and metal regulation it became desirable to re-investigate the occurrence and structure of goblet cells in lepidopterous larvae. Although goblet cells have a very similar basic structure, their appearance differs from species to species and in *Tineola* larvae, at least, there is a distinct dimorphism. In some species (*Deilephila, Galleria, Vanessa*) the goblet cells are reported to be fairly evenly distributed throughout the midgut. In others (*Pyrausta*) they are more numerous in the middle and posterior regions and in others again (*Dictyoploca*) they are more numerous in the anterior midgut (see Lotmar 1941). It is highly desirable, therefore, to establish whether the ability of *Tineola* larvae to digest wool and to maintain an unusually high pH and low oxidation-reduction potential in their midgut (Waterhouse 1952*b*) is associated with any unusual features of goblet cell structure or distribution.

II. Methods

Larvae of various instars of 18 species of Lepidoptera belonging to 17 Families were fixed in alcoholic Bouin, neutral formol alcohol, or Carnoy and generally stained in Bodian, Mallory, or haematoxylin. Bodian's 'Protargol' technique following alcoholic Bouin fixation was, in general, found to provide the best differentiation.

The following species were examined:

Heterocera

Tineidae *Tineola bisselliella* (Humm.)
Plutellidae *Plutella maculipennis* (Curtis)
Gelechiidae *Sitotroga cerealella* (Ol.)
Tortricidae *Tortrix postvittana* (Walk.)
Galleriidae *Galleria mellonella* (L.)
Phycitidae *Plodia interpunctella* (Hubn.)
Phycitidae *Ephestia kuhniella* Zell.
Arctiidae *Spilosoma canescens* (Butl.)
Anthelidae An unidentified species
Noctuidae *Dasygaster hollandiae* Guen.
Agarastidae *Phalaenoides glycine* Lew.
Sphingidae An unidentified species
Boarmiidae *Mnesampela privata* Guen.
Bombycidae *Bombyx mori* (L.)

Rhopalocera

Papilionidae *Papilio aegeus* Don.
Satyridae *Heteronympha merope* (Fabr.)
Nymphalidae *Pyrameis itea* (Fabr.)
Pieridae *Pieris rapae* (L.)

III. Results

A careful study was made of the goblet cells in *Tineola* larvae which will, therefore, be described in some detail. Other species will then be dealt with more briefly.

(*a*) *Histology of the Midgut of* Tineola *Larvae*

The general features of the histology of the larval midgut have been described by Lotmar (1941) and only the distribution and structure of the cell types will, therefore, be considered here. In the anterior region of the midgut (approximately the first quarter) goblet cells are so numerous that they are separated by no more than one or two columnar cells, and occasionally they appear to touch one another (Waterhouse 1952*a*, Fig. 1*A*, *B*). Then follows a middle region (about half the length of the midgut) in which goblet cells occur only infrequently and columnar cells predominate. This leads into the posterior region (the last quarter) in which goblet cells occur almost as frequently as in the anterior region. Small groups of regenerative cells are scattered along the entire midgut and lie near the basement membrane. These regenerative cells are inconspicuous following a moult, but become more numerous and more apparent as the next moult approaches.

The columnar cells in the middle region of the midgut (Waterhouse 1952*a*, Fig. 1*C*) have an appearance typical of simple epithelial cells which occur in the midgut of many insects. The nuclei lie at about the centre of the cell and each cell possesses a conspicuous, but low, striated border. On the other hand, owing to the presence of numerous goblet cells, the cylinder cells in the anterior and posterior regions are often narrow at the level of the nucleus and wider proximally and distally (Waterhouse 1952*a*, Fig. 1*B*). In these regions the striated border, although variable, is comparatively high.

The goblet cells of *Tineola* larvae do not conform in structure to past descriptions of goblet cells in other lepidopterous larvae, principally in the fact that the cavities of the cells do not appear to open into the lumen, even when the cells are examined in thin (4 μ) sections. The cells narrow as they approach the lumen, but may expand again a little to form a "cap" before they terminate apically with a sharp demarcating line. This line has the appearance of a bounding cell membrane. There is no sign of a striated border on the lumen boundary of the goblet cells. The tips of the goblet cells generally terminate level with the general line of the base of the striated border of the columnar cells (Waterhouse 1952*a*, Fig. 1*B*, *C*). However, they sometimes lie below this level (illustrated for *Galleria* in Plate 2, Fig. 6) communicating with the lumen by narrow passages between the higher columnar cells. Whatever their level in relation to the striated border, the goblet cells expose relatively little

surface to the gut lumen. The goblet cells in the anterior and posterior regions of the midgut differ in shape from those in the middle region (Waterhouse 1952*a*, Fig. 1*B*, *C*). In the anterior and posterior regions the cells are cigar-shaped, being fairly uniform in width until they start to narrow towards the gut lumen. If there is a slight variation in width, the maximum is reached in the distal half of the cell (Plate 1, Figs. 1 and 3). In the middle region of the midgut, the goblet cells are more flask-shaped, the basal portion of the cell being enlarged and the distal being reduced in diameter (Plate 1, Figs. 2, 4, 5, 6).

The basal quarter or fifth of each goblet cell contains the nucleus and dense cytoplasm similar to that of the columnar cells; the distal three-quarters of the cells is largely occupied by a centrally placed "cavity" with uniformly staining, structureless contents and, at times, some granules. The contents of the cavities (and the nuclei of the goblet, columnar, and regenerative cells) are heavily impregnated with silver following Bodian's technique. The cytoplasm of the columnar cells and the striated border is, by contrast, impregnated only very lightly (Plate 1, Figs. 1-6).

In the goblet cells of the anterior and posterior midgut the region of each cell bordering the basal half of the cavity has a different appearance from the remainder of the cytoplasm. This region sometimes appears to be faintly striated (Plate 1, Fig. 3) and evidently corresponds to the more definitely striated lining to the cavity of the goblet cells in some other lepidopterous larvae (see later). It has a different appearance and staining reactions from the striated border of the columnar cells.

In the goblet cells in the middle portion of the midgut, the region that stains heavily with Bodian often appears to extend around on either side of the nucleus towards the base of the cell (Waterhouse 1952*a*, Fig. 1*C*). This extension may either be uniform or take the form of a series of lobes invaginated into the cytoplasm. These lobes may be simple or branched (Plate 1, Figs. 4-6). Sections stained with aniline blue, safranin, or eosin show that the inner region of the Bodian-stained material constitutes the cavity of the cell. Occasionally there is some slight indication of striations in the outer region which, therefore, corresponds with the striated lining of other goblet cells.

(*b*) *Goblet Cells in Other Lepidoptera*

From Figure 1*A*, *B* and Plates 1-3 it can be seen that the goblet cells of a number of other species of lepidopterous larvae (both moths and butterflies) have a structure generally similar to the cigar-shaped goblet cells of *Tineola*. Principal variations occur in:

(i) The depth to which the goblet cavity extends into the cells. Although there is a certain amount of variability within a single species, the goblet cavity frequently occupies a characteristic position in the cell. It extends almost to the base of the cell in *Bombyx* (Plate 1, Fig. 11) and *Plutella* (Plate 1,

Fig. 12), but only to about the centre of the cell in *Plodia* (Plate 1, Fig. 10). Other species occupy intermediate positions.

(ii) The appearance of the lining of the goblet cavity. In most species the lining is only faintly striated, but in others, e.g. *Galleria* (Plate 2, Figs. 6 and 7), there are comparatively long and conspicuous filaments, somewhat reminiscent of the striated border of the columnar cells. In *Heteronympha* (Plate 1, Fig. 7) and *Plutella* (Plate 1, Fig. 12) striations can also be quite readily seen, although the "filaments" are more compact than in *Galleria.*

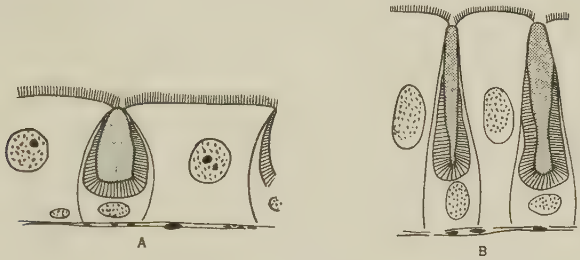

Fig. 1.—A, diagram showing goblet and columnar cells of *Plutella.* *B*, diagram showing goblet and columnar cells of *Heteronympha.*

(iii) The staining reactions of the goblet cavity. The goblet cavity of most species stains heavily with Bodian, but, in others, it stains comparatively lightly and the latter is usual in most species immediately after moulting. Furthermore, the tip of the goblet cell in some species (e.g. *Galleria, Plodia, Papilio aegeus*) is strongly fuchsinophilic, whereas in others there is very little red staining.

(iv) The accumulation of granular material at the tip of the cell. Heavily stained granular material at the lumen border of the cell was a conspicuous feature of most species (e.g. *Papilio aegeus* (Plate 2, Fig. 2) and *Pyrameis itea* (Plate 2, Fig. 5)), although it occurred less frequently in others.

(v) The diversity of goblet cell structure. No distinct dimorphism in goblet cell type (such as occurs in *Tineola*) has been observed in other species, although the latter have been studied far less carefully. The most striking changes are those that occur in different instars. In young larvae the epithelium is generally simple and the goblet cells and columnar cells are clearly separated. In later instars, in which the epithelium commonly becomes highly convoluted, the cells are relatively longer and narrower and the goblet cells frequently become crowded together. In the young larvae the goblet cavity is often wider than in the folded epithelium of more mature larvae. The change in appearance with advancing instar is exemplified in the photographs of young *Papilio aegeus* (Plate 2, Figs. 1 and 2) and a mature larva (Plate 2, Figs. 3 and 4).

(vi) The ratio of goblet to columnar cells. In different regions of the midgut it is common to find the ratio of goblet cells to columnar cells varying

from 1 : 1 to 1 : 5. Even when the goblet cells are fairly widely spaced two contiguous cells may occur (*Plodia*, Plate 1, Fig. 10). Where the epithelium is highly convoluted in later instars the crowded goblet cells appear to touch each other (Plate 2, Fig. 4). However, they must, in reality, generally be separated by columnar cells, whose nuclei (lying about the centre of the epithelium) and striated border are clearly visible.

(vii) The position of the goblet cell in relation to the folds of the epithelium. In some species (*Tortrix*, Plate 3, Figs. 7 and 8) the goblet cells occur most commonly at the bases of the epithelial folds. However, they may occur in all positions (*Pyrameis itea*, Plate 2, Fig. 5) and are shown on the side (*Diacrysia*, Plate 3, Fig. 4 also at base, Plate 3, Fig. 3) and at the top (anthelid, Plate 3, Fig. 6) of epithelial folds.

IV. Discussion

One of the striking features of this brief survey of goblet cell structure is the general similarity of these cells in most species. The cells that deviate most from the basic pattern are those occurring in the middle region of the *Tineola* midgut. It might be expected, therefore, that goblet cells will have a similar function in all lepidopterous larvae.

It appears that, very largely by analogy with the mucus-secreting goblet cells in the digestive tract of vertebrates, the cavities of the goblet cells have been considered to discharge their contents through an opening that communicates directly with the gut lumen. Except where fixation has been faulty, no such openings have been seen in the sections on which the present study was based and it is noteworthy that many authors have not figured any direct opening, although even they have not suggested that the cavity was closed. Very thin (3-4 μ) sections of several species (e.g. *Tineola, Plutella*) failed to demonstrate any direct passage, so that, if such does occur, it must either have a diameter less than 3 μ or follow an oblique path into the lumen. Under such circumstances, no direct passage would be seen. However, no passages could be identified in horizontal sections where channels as small as 0.5 μ in diameter should be visible. There is some functional evidence that, if any discharge of material from goblet cells occurs, this must take place through a membrane or, at most, through a very narrow passage, which appears to be readily blocked. This evidence is derived from the accumulation of the sulphides of many metals in the goblet cavities of *Tineola* larvae. These sulphides, together with ionized iron and copper compounds, are accumulated from metal-enriched diets and persist, after transfer of larvae to a normal diet, until the next moult, when the entire epithelium is cast off and regenerated (Waterhouse 1952*a*). Dyes and pH and oxidation-reduction indicators are also accumulated in the goblet cavities, although some of the more soluble pH indicators (e.g. phenol red) disappear before moulting if the larvae are transferred to a control diet (Waterhouse 1952*b*). Several authors have recorded what appears to be the production of secretions by the goblet cells (see Lotmar 1941), but it remains

to be shown that this is not the result of faulty histological technique. If there were a continuous discharge of material from the goblet cavity through a passage into the lumen it is probable that any accumulated metallic salts would be discharged also. On the other hand, sparingly soluble materials would be retained if secretion occurred through a cell membrane, although the soluble dyes would be discharged without difficulty. The massed, densely staining, granular material, which has been stated to occur in the tips of many goblet cells of larvae feeding on normal diets would similarly occlude, under natural conditions, any passage leading from the cavity into the gut lumen. It is relevant to recall that, in larvae of *H. merope* feeding on grass, the goblet cell cavities often contain dark brown material, particularly in the middle region of the midgut (Waterhouse 1952*a*). This brown material is presumably accumulated from the food just as are dyes in *Tineola* larvae. Finally it is clear from the careful studies of Lotmar (1941) on the formation of the new midgut epithelium in *Tineola* at moulting that the goblet cavity is formed within the developing cell, so that it has clearly not arisen by invagination of the lumen border. This would explain also the different appearance and staining properties of the striated lining to the cavity and the striated border of the columnar cells. These have frequently been considered of similar origin, although the differences in properties have been clearly recognized.

No evidence was obtained of replacement of goblet or columnar cells between moults. However, cigar-shaped, black masses were occasionally seen between the epithelium and the peritrophic membrane of *Tineola* larvae feeding on a diet rich in nickel (Waterhouse 1952*a*). This may have been the discharge of the massed accumulations of nickel sulphide from a few of the goblet cavities or, alternatively, a casting off of a few entire goblet cells following disruption of function due to blockage with nickel sulphide deposits.

The only demonstrated function of goblet cells is in metal and dye storage and it appears that they play an important role in storage excretion (Waterhouse 1952*a*, 1952*b*). Because of the small surface they expose to the gut lumen it would not be anticipated that they would play an important part in absorption. However, they must be capable of absorption, unless, as appears more likely, the metals are first absorbed by the adjoining columnar cells and subsequently transferred laterally to the goblet cells for storage.

Goblet-like cells have been reported to occur in the Thysanura and Ephemeroptera and true goblet cells in Trichoptera (Shinoda 1927). This report remains unconfirmed and appears to have very doubtful validity. A careful examination of an extensive series of Bodian-stained sections of *Ctenolepisma longicaudata* and *Heterojapyx evansi* (Thysanura) and of more than one species of larval Ephemeroptera showed no sign of goblet-like cells in the midgut and this is in agreement with published observations on other species of these orders. Certainly, therefore, Shinoda's statement cannot be regarded as a true generalization for the first two orders. Furthermore, goblet cells could not be detected in the course of a careful examination of representatives of three families of Trichoptera (Glasgow 1936). Sections of the midgut of larvae

of an unidentified species of Trichoptera were examined after staining with Bodian, but no goblet cells could be found. Since the species examined by Shinoda (*Philopotamus* sp., Fam. Philopotamidae) belongs to the same superfamily as two of the families examined by Glasgow, Shinoda's report of the presence of goblet cells must be treated with great reserve until it is confirmed.

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Explanation of Plates 1-3

Plate 1

All sections stained with Bodian

Fig. 1.—Anterior midgut of *Tineola* larva, showing cigar-shaped goblet cells alternating with columnar cells.

Fig. 2.—Middle region of midgut of *Tineola* larva, showing flask-shaped goblet cells.

Fig. 3.—Cigar-shaped goblet cell of *Tineola* larva, showing striated lining to base of cavity.

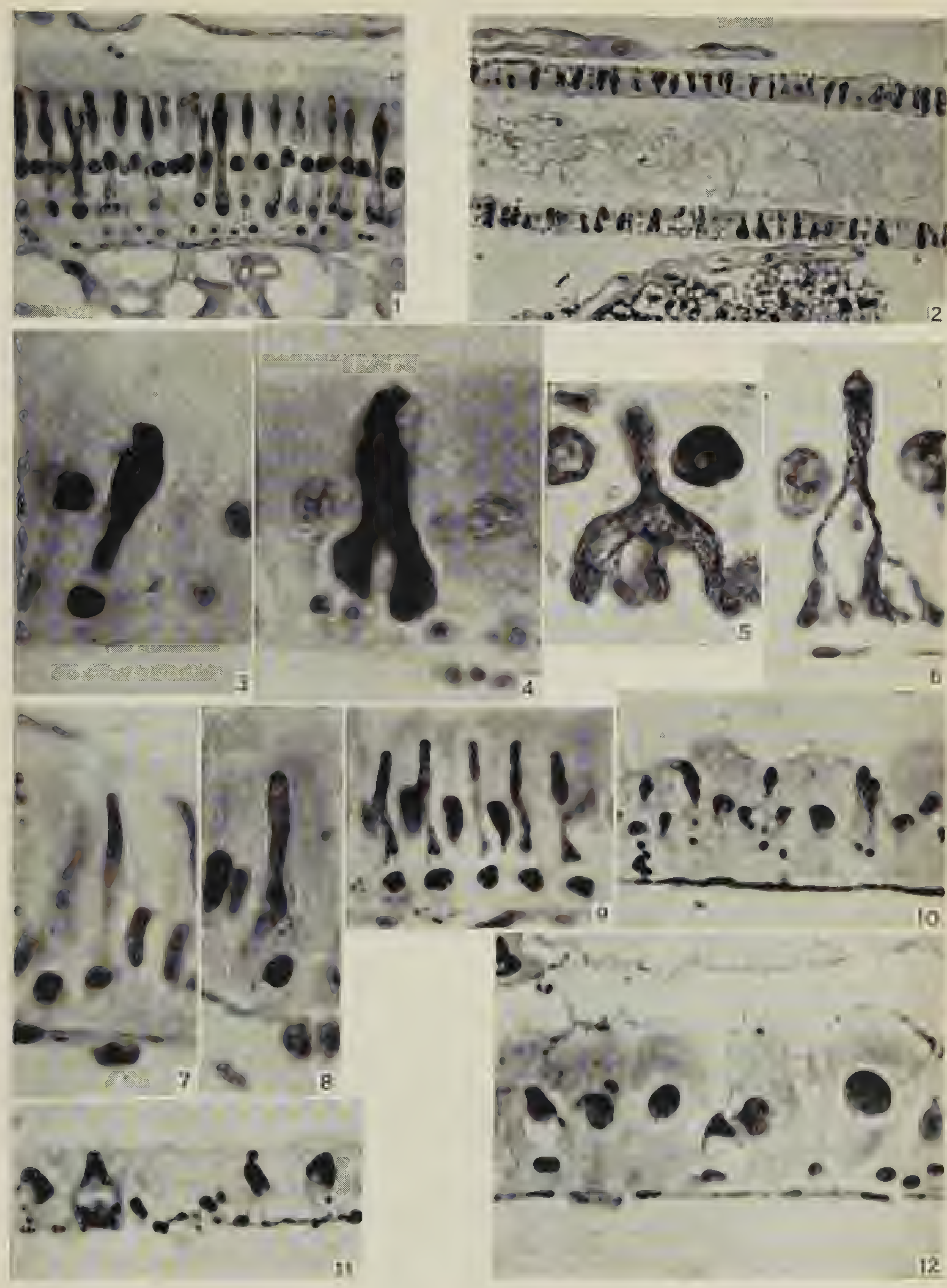
Fig. 4.—Flask-shaped goblet cell of *Tineola*, showing cavity extending around the basally situated nucleus.

Figs. 5 and 6.—Flask-shaped goblet cell of *Tineola* larva, showing lobes of cavity invaginated into cytoplasm.

Fig. 7.—Goblet cell of *Heteronympha* larva, showing striated lining to cavity.

Figs. 8 and 9.—Typical goblet cells of *Heteronympha* larva.

DIGESTION OF WOOL BY INSECTS. V



DIGESTION OF WOOL BY INSECTS. V

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Fig. 10.—Goblet cells of *Plodia* larva.
Fig. 11.—Goblet cells of young *Bombyx* larva.
Fig. 12.—Goblet cells of *Plutella* larva, showing striated lining to cavity.

PLATE 2

All sections stained with Bodian

Fig. 1.—Goblet cells of young *Papilio aegeus* larva.
Fig. 2.—Goblet cell of young *P. aegeus* larva at higher magnification, showing densely staining cap.
Fig. 3.—Goblet cell of mature *P. aegeus* larva at high magnification, showing densely staining cap.
Fig. 4.—Goblet cells of mature *P. aegeus* larva.
Fig. 5.—Goblet cells of *Pyrameis itea* larva.
Figs. 6 and 7.—Goblet cells of young *Galleria* larva.

PLATE 3

Figs. 1 and 2.—Goblet cells of *Pieris* larva, Bodian.
Fig. 3.—Goblet cells of *Diacrysia* larva, Mallory.
Fig. 4.—Same, Bodian.
Fig. 5.—Goblet cells of *Phalaenoides* larva, Mallory.
Fig. 6.—Goblet cell of anthelid larva, Bodian.
Figs. 7 and 8.—Goblet cells of *Tortrix* larva, Bodian.

STUDIES ON THE DIGESTION OF WOOL BY INSECTS

VI. THE pH AND OXIDATION-REDUCTION POTENTIAL OF THE ALIMENTARY CANAL OF THE CLOTHES MOTH LARVA (*TINEOLA BISSELLIELAL* (HUMM.))

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Summary

The pH and oxidation-reduction potential of the contents of the alimentary canal of *Tineola* larvae were determined by adding suitable indicators to their food.

The contents of the foregut and the introductory portion of the midgut have a pH of 8.0 to 8.4. Then follows a region of pH 8.5 to 9.0, which leads into the middle region of the midgut with a pH of 9.8 to 10.0. In the posterior midgut the pH drops first to 8.5 to 9.0 and then to 7.8 to 8.0. The hindgut contents have a pH of 4.6 to 5.8.

The oxidation-reduction potential of the foregut and introductory region of the midgut lies in the range —20 to +32 mV. Then follows a region with a potential of about —200 mV. The middle region has a potential in the range —250 to —280 mV. In the posterior midgut the potential rises first to —150 to —190 mV. and then to above +62 mV. The hindgut contents have a potential higher than +250 mV.

The pH of the contents of the goblet cell cavities in the anterior and posterior regions is 6.2 to 6.5 and the potential +31 to +80 mV. Because these values are very different from those of the contents of the digestive tract it is unlikely that the goblet cells function principally in the production and accumulation of digestive secretions. It is suggested, instead, that important functions of these cells are storage and active excretion. These functions may be correlated with the absence in Lepidoptera of replacement of epithelial cells between moults, a process characteristic of many other insects.

I. INTRODUCTION

The current theory of the mechanism of digestion of wool by larvae of the clothes moth *Tineola* is that the alkaline, highly reducing, midgut digestive juices reduce the disulphide bonds of wool cystine to sulphydryl groups. This reduced wool is far less resistant than normal wool to enzymic attack and is degraded by the larval proteolytic enzymes (Linderstrøm-Lang and Duspiva 1936). Some other animals, which do not possess the capacity to reduce wool (and hence cannot digest it) can, however, digest previously reduced wool. It is clear, therefore, that, for the theory of the mechanism of digestion to be soundly based, detailed information is required on the pH and oxidation-reduction potential of the digestive tract of *Tineola* larvae.

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Many years ago Sitowski (1905) reported that the foregut and midgut contents of clothes moth larvae were alkaline to litmus, but that the hindgut contents were acid. Hindgut acidity was due to the presence of an organic acid (uric acid), since congo red failed to turn blue. These results were confirmed and extended by Titschack (1922) who, in addition, reported that phenolphthalein took on its alkaline coloration in the midgut, thereby indicating a pH greater than about 9.7. Confirmatory tests were also reported by Schulz (1925) using litmus. In 1936, Duspiva re-investigated the problem. He found, using thymol blue, that the midgut secretions were more alkaline than pH 9 and by glass electrode measurements that they averaged 9.9, with a range in different larvae from 9.44 to 10.15. Fore- and midgut alkalinity and hindgut acidity are, therefore, well established. However, it was considered desirable to learn more about the variation in pH along the digestive tract and, in particular to define the zone of extremely high pH by means of a wider range of pH indicators than previously employed. Furthermore, information was also required on the pH of the goblet cavities, which have been held to act as reservoirs for digestive enzymes (see Waterhouse 1952*b*).

Linderstrøm-Lang and Duspiva (1936) determined the oxidation-reduction potential of the contents of the alimentary canal to be in the vicinity of -300 mV. by feeding six redox indicators. They also recorded dye uptake by the midgut epithelium. The results of Day (1951*a*) with triphenyltetrazolium chloride confirmed the existence of reducing conditions in the midgut although the results obtained were rather confusing. The dye experiments are extended in the present series of tests, which indicate that the conditions in the most reducing region of the midgut are probably slightly less reducing than previously thought. They also provide the first information on the potential in other regions of the gut and in the goblet cell cavities.

II. Methods

Larvae of *Tineola bisselliella* were transferred after feeding for 3-4 weeks on a casein-yeast diet at 27°C. to woollen fabric which had been impregnated with saturated aqueous solutions of indicators. A small amount of alkali was required to bring some of the pH indicators into solution. At times indicators were also fed with silk and with the yeast-casein diet.

Larvae were examined after one or two days on these treated diets and dissected, where necessary, to determine more accurately the regions of colour change. Major differences in coloration could be seen quite readily through the transparent body wall of the living larva. Because of the solubility of the indicators in fluids used for histological preparations no sections were prepared except of methylene-blue-fed larvae. Methylene blue was rendered insoluble by the ammonium molybdate procedure. pH indicators of the sulphonphthalein series,* which are relatively free from salt and protein errors

* BDH water-soluble indicators.

were used except for the range above pH 9.0, where recourse had to be made to other indicators to extend the range. The presence of the reduced leuco form of some of the oxidation-reduction indicators was confirmed by exposing the gut to oxidation by air following dissection.

III. Results

(*a*) pH

Table 1 lists the pH indicators that provided useful information when fed to *Tineola* larvae and Figure 1 illustrates the results obtained. The contents of the foregut and the short introductory portion of the midgut had a pH of 8.0-8.4. Then followed an equally short second region of the midgut with alkalinity increasing to about pH 9. Commencing with the middle region of the midgut (Waterhouse 1952*a*, Fig. 1) the alkalinity rose sharply to pH 9.8-10. From the end of the middle region and throughout the posterior midgut the alkalinity decreased, first to pH 8.5-9.0 and then to 7.8-8.0. In the hindgut the contents had a pH of 4.6-5.8.

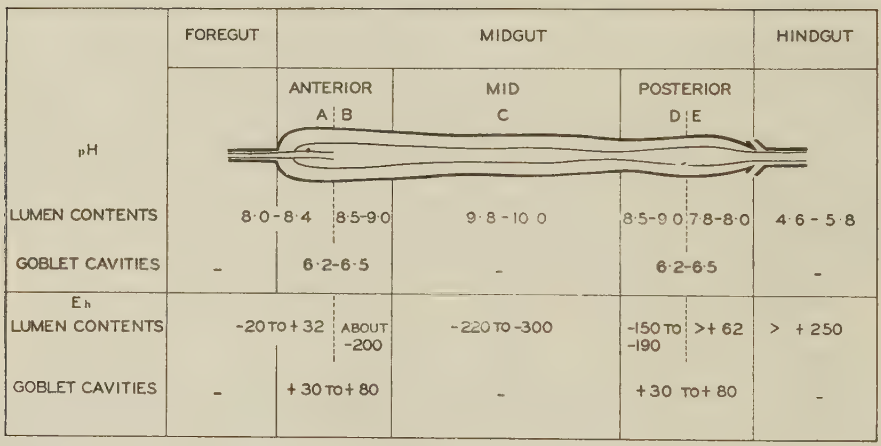

Fig. 1.—Diagram showing pH and oxidation-reduction potential (mV.) of various regions of the digestive tract of *Tineola* larvae.

In the alimentary canal epithelium pH indicators were present in sufficient concentration to be visible only in the cavities of the cigar-shaped goblet cells of the anterior and posterior regions. No accumulation was observed in the flask-shaped goblet cells of the middle region (Waterhouse 1952*a*). The contents of the cigar-shaped goblet cell cavity had a pH of 6.2 to 6.5. When larvae that had accumulated many of the pH indicators in the goblet cavities were transferred to control fabric the accumulations disappeared in the course of one or two days, indicating that these accumulations are not static, unlike those of the metal sulphides (Waterhouse 1952*a*).

(*b*) *Oxidation-Reduction Potential*

The oxidation-reduction potential indicators used and the potentials recorded in the lumen of the digestive tract are shown in Table 2 and Figure 1. The potential at which these indicators are 50 per cent. oxidized (their E'_0

TABLE 1

pH OF THE CONTENTS OF THE ALIMENTARY CANAL AND GOBLET CELL CAVITIES OF *TINEOLA* LARVAE

	Gut Contents							Goblet Cavity Contents		
		Midgut Region (See Fig. 1)								
Indicator	Foregut and Midgut A	*B*	*C*	*D*	*E*	Hindgut		*A* + *B*	*C*	*D* + *E*
Brom-phenol blue	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0		>4.0		>4.0
Brom-cresol green	>4.6	>4.6	>4.6	>4.6	>4.6	>4.6		>4.6		>4.6
Chlor-phenol red	>5.8	>5.8	>5.8	>5.8	>5.8	<5.8		>5.8		>5.8
Brom-cresol purple	>6.2	>6.2	>6.2	>6.2	>6.2	<6.0		>6.2		>6.2
Brom thymol blue	>6.9	>6.9	>6.9	>6.9	>6.9	<6.5		<6.5		<6.5
Phenol red	>7.6	>7.6	>7.6	>7.6	>7.6	<7.2		<7.6		<7.6
Cresol red	>7.8	>8.0	>8.0	>8.0	>7.8	<7.6		<7.6		<7.6
Meta-cresol purple	>8.0	>8.0	>8.0	>8.0	<7.8	<7.8		>7.6-<7.8		<7.6
Thymol blue	<8.4	8.4-8.8	>9.0	8.4-8.8	<8.4	>8.4		<8.0		<8.0
Phenol violet	<8.5	About 9.0	>9.5	About 9.0	<8.5	<8.5		<8.5		<8.5
O-cresol-phthalein	<9.0	<9.0	>9.5	<9.0	<9.0	<9.0				
Phenol-tetrachloro-phthalin	<8.2	>8.5	>9.0	>8.5	<8.2	<8.2				
Phenol-phthalein	<8.3	<9.0	>9.7	<9.0	<8.3	<8.3				
Phenol-thymol-phthalein	<9.4	About 9.6	>9.8	About 9.6	<9.4	<9.4				
BDH universal indicator	About 8.0	8.5-9.0	9.5-10.0	8.5-9.0	About 8.0	6.0-6.5		About 6.5		About 6.5
Range	8.0-8.4	8.5-9.0	9.8-10.0	8.5-9.0	7.8-8.0	4.6-5.8		6.2-6.5		6.2-6.5

value) varies with pH (with the exception of benzyl viologen (Michaelis and Hill 1933)) and it is to be expected therefore that different potentials will be recorded for each of the six zones of varying pH. The E'_0 values of the

TABLE 2

OXIDATION-REDUCTION POTENTIAL OF THE CONTENTS OF THE ALIMENTARY CANAL OF *TINEOLA* LARVAE

Indicator	E'_0 (V.) at				Colour Change (oxidized to reduced)	Foregut and Midgut *A*	Midgut Region (see Fig. 1)				Hindgut
	pH 5	8	8.8	10			*B*	*C*	*D*	*E*	
1-naphthol 2-sulphonate indophenol	*c.* + 0.250	+ 0.062	+ 0.010	— 0.055	Pink to colourless	Colourless	Colourless		Colourless	Pink > + 0.062	Pink > + 0.250
Toluylene blue	+ 0.0221	+ 0.082	+ 0.057		Blue to colourless	Colourless	Colourless		Colourless		Blue
Thionine	+ 0.138	+ 0.032	+ 0.008	— 0.025	Blue to colourless	Colourless < + 0.032	Colourless		Colourless	Light blue	Blue
Methylene blue	+ 0.101	— 0.020	— 0.044	— 0.08	Blue to colourless	Blue > — 0.020	Colourless		Colourless	Light blue	Blue
Indigo tetrasulphonate	+ 0.065	— 0.083	— 0.108	— 0.13	Blue to yellow	Blue or greenish blue	Yellow		Yellow	Blue	Blue
2,3,5-triphenyltetra-zolium chloride		(— 0.080 at pH 7)			Colourless to red	Pink	Pink		Pink	Pink	
Indigo trisulphonate	+ 0.032	— 0.121	— 0.146	— 0.175	Blue to yellow	Blue to green	Yellow		Green < — 0.146	Blue	Blue
Indigo disulphonate	— 0.010	— 0.167	— 0.193	— 0.22	Blue to yellow	Light blue	Greenish yellow — 0.220 to — 0.300		Bluish green > — 0.193	Blue	Blue
Janus green		(— 0.225 at pH 7)			Blue to colourless	Pink	Pink		Pink	Pink	Pink
Phenosafranin	— 0.160	— 0.259	— 0.295	— 0.350	Pink to colourless	Pink	Pink		Pink	Pink	Pink
Rosindulin	— 0.161	— 0.340	— 0.385	— 0.438	Red to colourless	Dark pink	Light pink		Dark pink	Dark pink	Dark pink
Benzyl viologen	— 0.359	— 0.359	— 0.359	— 0.359	Colourless to violet	Colourless	Colourless		Colourless	Colourless	Colourless
					Range	— 0.020 to + 0.032	About — 0.200	— 0.220 to — 0.300	— 0.150 to — 0.190	> + 0.062	> + 0.250

dyes at different pH values are taken from Butler (1935), Linderstrøm-Lang and Duspiva (1936), Michaelis (1931), and Hewitt (1950).

The contents of the foregut and introductory region of the midgut had a value near zero potential (in the range —20 to +32 mV.). The short second region of the anterior midgut was considerably more reducing (—200 mV.). Commencing with the middle region of the midgut there was a further decrease in potential, which reached its lowest level in the digestive tract (—220 to —300 mV.) in this region. At the beginning of the posterior midgut the potential first rose to —150 to —190 mV. and then to +62 mV. or higher. In the hindgut the conditions were oxidizing (at least +250 mV.).

TABLE 3

OXIDATION-REDUCTION POTENTIAL IN THE MIDGUT EPITHELIUM OF *TINEOLA* LARVAE. COLOUR CHANGES OF THE DYES ARE SHOWN IN TABLE 2

Indicator	E'_0 at pH 6.4	Eh of Goblet Cavities	Midgut Region (See Fig. 1)			
			A	*B*	*C*	*D* and *E*
1-Naphthol 2-sulphonate indophenol	+ 0.159		No colour			
Toluylene blue	+ 0.141		No colour			
Thionine	+ 0.080	< + 0.80	Very occasionally turns blue on oxidation		No colour	
Methylene blue	+ 0.031	> + 0.031	Deep blue in goblet cells: few blue granules in columnar cells	Colourless	Sometimes very light blue	
Indigo tetrasulphonate	— 0.017	> — 0.017	Light blue in goblet cavities; general diffuse blue		No colour	
2,3,5-Triphenyl-tetrazolium chloride	> — 0.181		Entire epithelium pink			
Indigo trisulphonate	— 0.051	> — 0.051	Goblet cavities blue			
Indigo disulphonate	— 0.092	> — 0.092	Goblet cavities blue			
Janus green	> — 0.225	> — 0.225	Purple, mainly in goblet	Diffuse salmon		
Phenosafranin	— 0.235	> — 0.235	Entire epithelium a light pink			
Rosindulin	— 0.245	> — 0.245	Faint pink in goblet cells			
Benzyl viologen	— 0.359	> — 0.239	Colourless			

Unlike the pH indicators, some redox indicators were accumulated in detectable amounts in the goblet cells of all regions and, to a lesser extent, also by the columnar cells (Table 3). Although it is not possible in the absence of sections to establish the precise location of the indicators, they generally appeared to be accumulated in the goblet cavities. However, sections did indicate that fully coloured methylene blue occurred principally in the cytoplasm of

the anterior goblet cells and in the lining of the goblet cavities of the middle region. Small scattered granules of dye also occurred in the columnar cells (Waterhouse 1952*a*).

From available data a potential within the range +30 to +80 mV. is indicated in the anterior and posterior goblet cells. Because of the absence of information on the pH of the contents of the goblet cavities in the middle region of the midgut it is not possible to assign a potential value to them. Nevertheless some inferences can be drawn from the facts that indigo trisulphonate is fully reduced in the lumen of the mid midgut (Table 2), but is oxidized in the goblet cell cavities of this region (Table 3). This suggests that the potential in the cavities is higher than that in the lumen. An alternative possibility is that the pH of the cavity contents is considerably higher than that of the lumen of the gut (i.e. greater than 10.0). It is conceivable that the goblet cells of this region produce the very alkaline secretion which is responsible for the high midgut alkalinity, although their relatively infrequent occurrence in this region suggests that this is unlikely. If they do produce a secretion having a pH of 10 or higher, they certainly differ very markedly in function from the goblet cells of the anterior and posterior regions to which they show some functional resemblance in accumulating (albeit less frequently and less readily) metal sulphides (Waterhouse 1952*a*) and redox indicators.

IV. Discussion

Although the very much greater precision of electrical methods over the indicator method for measuring pH is clear, nevertheless the value of the indicator method is well illustrated in the present experiments. It is now possible to say, for example, that only in the middle region of the midgut is a pH as high as 10.0 attained. In fact, it is rather surprising that pooling the contents of the anterior and posterior midguts with those of the middle region, as was done for the glass electrode measurements of Duspiva (1936), still gave an average pH reading of 9.9. It is possible, therefore, that the pH of the middle region actually lies between 10 and 11, in which range it is not possible to determine the pH more accurately with available indicators.

One rather unexpected feature of the pH records is the value of 8.0-8.4 for the foregut contents, even when wool having a pH of 6-7 was fed. Unless it can be explained on the basis that the salivary secretion is copious and alkaline, it would appear that fluid from the midgut is passed forward regularly into the foregut. The well-developed oesophageal invagination (Waterhouse 1952*a*, Fig. 1) apparently does not preclude this movement. Alkaline foregut contents can be observed before dissection in the feeding larva and during dissection the forward movement of fluid from the beginning of the midgut into the foregut can sometimes be observed.

The materials responsible for midgut alkalinity, which is characteristic of all Lepidoptera (Waterhouse 1949), have not yet been identified. However, it was suggested many years ago (Kirkland and Smith 1898) that potassium

phosphate was largely responsible for the alkalinity of the midgut of the gypsy moth larva. This view receives support from Japanese workers on *Bombyx* larvae who found that the ash of the digestive juices contains 46 per cent. K_2O, 35 per cent. Na_2O, and 5 per cent. P_2O_5 (Itaya 1936). Furthermore, relatively large amounts of inorganic phosphate have been reported to occur in *Deilephila euphorbiae* (Heller 1949; Heller, Karpiak, and Zubikowa 1950).

Hindgut acidity is due, partly at least, to the presence of uric acid and urates which form some 30-40 per cent. of the weight of the faeces (Holland and Cordebard 1926; Powning, unpublished data). Since approximately the same pH is maintained on a diet of silk or yeast and casein as on wool, gut pH is not greatly influenced by the nature of the food. Silk is not digested by the larva although it is ingested. On this diet hindgut acidity is probably maintained by excretion of uric acid produced by larval fasting metabolism.

The establishment of very reducing conditions only at the posterior end of the anterior midgut agrees well with the observations that visible digestion of wool fibres can first be detected with polarized light in this region and that this region is poorly tracheated compared with most other insects (Day 1951*a*, 1951*b*). The precise value of the oxidation-reduction potential in the middle region of the midgut, if indeed it is accurately poised, is of some interest since it influences the selection of possible systems that may be responsible for its maintenance. The fact that gallophenine shows its oxidized coloration in this region (Table 14 of Linderstrøm-Lang and Duspiva 1936) and that indigo disulphonate is partly (probably more than 50 per cent.) reduced (Table 2; see also Linderstrøm-Lang and Duspiva 1936) suggests that the potential (if the pH is 10) lies somewhere between —220 and —290 mV., and probably in the range —250 to —280 mV. Additional accuracy will be possible only when further indicators are available in this range. Whereas this range is little different from "in the neighbourhood of —0.3 volts" (Linderstrøm-Lang and Duspiva 1936), it is of value to indicate that the potential is probably somewhat more positive, rather than more negative, than —300 mV. Since the potential of the cystine-cysteine system is —350 mV. at pH 10 (Fruton and Clarke 1934) it appears that this system is not alone responsible for the maintenance of the reducing potential in the middle region of the midgut, although sulphydryl groups are abundant in the food undergoing digestion. It is of interest to record that xanthine oxidase has been detected in the midgut of *Tineola* larvae (Day 1951*b*), since the hypoxanthine-uric acid reaction has a very low oxidation-reduction potential (Green 1934).

The value of +250 mV. or higher for the hindgut contents, which are rich in uric acid, is somewhat surprising at first sight in view of the fact that uric acid has some reducing properties. However, the high positive potential is partly the result of the low pH (4.6-5.8) and would probably not be influenced much by the small amount of uric acid in solution. It might be influenced more by any soluble urates present. The hindgut is not particularly richly tracheated, although it is relatively better supplied than the midgut.

Some information on the probable function of the goblet cells can be derived from the present tests. In the anterior and posterior regions of the midgut the contents of the cavities have a pH (6.2-6.5) considerably lower than that of the lumen contents (7.8-9.0). Certainly in the foregut, therefore, the goblet cavity contents cannot be responsible for the maintenance of an alkaline pH in the digestive tract. Furthermore, the cavity contents have an oxidation-reduction potential more oxidizing than the mass of food undergoing digestion. If, therefore, the contents play any part in the maintenance of the potential in the lumen of the gut, it must be in the production of precursors of the systems respcnsible rather than in the production of the actual systems operative. However, in view of the fact that the goblet cells occur least frequently in the middle region of the midgut, where the potential is lowest and the pH highest, it appears improbable that they play any important role in the maintenance of these conditions. There is good reason to believe therefore that, just as in other insects, the columnar cells function actively both in secretion of digestive enzymes and poising and buffering agents and in absorption.

The reason that goblet cells have been regarded as secretory is partly because of their superficial resemblance to the mucus-producing goblet cells of vertebrates and partly because they expose so little of their surface to the lumen that they would not be expected to be effective in absorption. Although there is no information available, there is no more reason to believe that the cavities are reservoirs for the accumulation of digestive enzymes than for enzymes responsible for maintenance of gut potential or for materials responsible for maintenance of gut pH. Other possibilities are that the goblet cells are specialized for intermediary metabolism or that they are primarily concerned with storage or active excretion. The presence of a cavity is difficult to reconcile with the specialized functions of intermediary metabolism, but is not at all inconsistent with the latter suggestions. Furthermore, the goblet cells have been demonstrated to have an important function in storage excretion, not only under the somewhat unusual conditions of high metal and dye intake, but also under normal conditions, such as in storing the brown material accumulated in the goblet cells of grass-fed *Heteronympha* larvae (Waterhouse 1952*a*).

With regard to active excretion the observation is relevant that masses of nickel sulphide, which by their shape had evidently accumulated in the goblet cell cavities, could occasionally be seen in the gut lumen of *Tineola* larvae (Waterhouse 1952*a*). This suggests either the discharge of solid material from the cavities, or the casting off of entire goblet cells under certain conditions. Furthermore, the disappearance of some accumulated pH indicators from the goblet cavities after transfer to control fabric indicates that movement of materials out of the goblet cavities occurs in the feeding larva. The fate of these indicators was not determined. However, as it is not improbable that they were discharged into the gut lumen, both active and storage excretion can probably be regarded as among the important functions of the goblet cells of Lepidoptera.

Perhaps goblet cells have been developed in conjunction with the method of epithelial regeneration in Lepidoptera. In this order no inter-moult replacement is apparent. Either the entire epithelium is cast off and completely regenerated at each moult or it is not completely degraded but is largely reorganized (Lotmar 1941). On the other hand, there is no reason to believe that the malpighian tubules of lepidopterous larvae are any less efficient in excretion than those of other insects. Furthermore, an exclusively excretory function does not adequately explain the frequent occurrence of goblet cells in some regions and their sparse occurrence in others, unless their density is, perhaps, related to the amount of absorption and intermediary metabolism proceeding in the various regions.

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DIGESTION OF WOOL KERATIN BY PAPAIN-BISULPHITE-UREA AND RELATED SYSTEMS

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Summary

The digestion of wool in papain-bisulphite solutions is greatly enhanced by the addition of urea,† reaching completion within a few hours at 50° or 70°C. Both wool digestion and uptake of papain by wool from urea solutions proceed optimally at pH 7.

Urea-bisulphite solutions containing other proteinases such as ficin or bromelin, which are activated by reducing agents, partially digest wool at pH 7, and similar solutions containing pepsin partially digest wool at pH 3, but mould protease, trypsin, beef liver cathepsin, and pig pancreas extract display little activity in comparison with papain at all pH values between 5.7 and 9.6.

Replacement of sodium bisulphite in the digestion mixture with other sulphur-containing reducing agents reduces the amount of wool digestion at pH 7. Of a series of compounds related to urea, which were tested at equimolecular concentrations in the presence of papain and bisulphite, thiourea assisted digestion to a greater extent than urea, but the other compounds were less effective. Guanidine is only slightly inferior to urea, but partial or complete replacement of an amino group in the urea molecule, as in methyl urea and formamide respectively, lowers the activity greatly.

Horn and feather keratin and skin collagen are readily digested by papain-bisulphite-urea but silk fibroin and plasma fibrin are less affected.

I. Introduction

Because of its pronounced resistance to attack by proteolytic enzymes, wool is much less readily damaged by living organisms, such as bacteria, moulds, and insects, than are most other protein materials. Under some conditions this resistance is overcome and it is important to identify such conditions and to determine the nature of the attack if means are to be sought for protecting the fibres from biological damage. Moreover, to prepare solutions and dispersions of wool of a type suitable for certain chemical studies, it is preferable to imitate the relatively mild enzymic digestion employed in nature rather than employ the more drastic and less specific chemical methods, for the former method is more likely to yield fragments of wool keratin in solution resembling intact keratin. For these reasons, particular interest attaches to the previously reported slow digestion of wool in solutions containing papain and bisulphite, which is the basis of the shrinkage resistance process developed by Middlebrook and

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† In this paper the term "digestion" signifies solution of wool through enzyme, physical, or chemical action without reference to the nature or fate of the substances removed from the fibres.

Phillips (1941). After 17 hr. incubation they found that the wool was reduced to a mass of cells, and H. Lindley (private communication) has observed complete digestion of the cortical cells from processed wool after prolonged incubation in papain-bisulphite solution at 65°C. In the present paper it will be shown that the addition of urea to the papain-bisulphite greatly assists attack on the wool to the stage of almost complete digestion within a few hours at 50° or 70°C. Wool is also extensively digested during incubation in other solutions containing similar combinations of sulphide-activated plant protease, or pepsin, reducing agent, and a compound structurally related to urea.

In some respects the present study is related to the work of Jones and Mechan (1943). These workers showed that 52 per cent. of wool substance was dissolved during 17 hr. incubation at 40°C. in 10M urea and 0.3M sodium bisulphite, but the remainder of the wool fibre was unattacked under these conditions.

II. Materials and Methods

(*a*) *Preparation of Wool*

The tips were removed from the staples of 50 lb. of 64's quality merino fleece and the remainder was extracted with ethanol for 7 hr. in a large brass Soxhlet apparatus, then immersed on four successive days in tap water, changing the water twice daily. On the fifth day distilled water was used and the wool was then squeezed well and dried in a current of warm air. Seeds and other impurities were removed from the scoured wool by hand-picking, and the product was stored in sealed metal containers.

(*b*) *Proteinases and Reagents*

The papain used was a high quality commercial grade grown in Ceylon and supplied in the form of a slightly brown powder. Analysis of aqueous extracts showed that the powder contained 12 per cent. soluble ash. Rapid digestion was confirmed in some tests using samples from two other sources. The trypsin was from Allen and Hanbury, and the pepsin used was a "Difco" product. The mould protease powder also contained other enzymes from *Aspergillus oryzae* (Crewther and Lennox 1950), and was prepared from culture filtrate by low temperature precipitation with ethanol and freeze-drying. Crude bromelin was prepared by disintegrating pineapple tissues in the Waring Blendor, separating the juice by centrifuging and filtering, adding five volumes of ethanol at 2°C., centrifuging to recover the precipitate, drying in a vacuum over calcium chloride, and grinding the dry residue. Crude ficin powder was prepared by collecting in ethanol the latex from freshly picked green figs of the tree *Ficus glabrata* and treating the precipitate as described for bromelin. The ficin used in the experiments reported in Tables 4 and 5 was purchased from the Delta Chemical Works, U.S.A. Cathepsin was prepared from beef liver by the method of Anson (1936), the product obtained in the final dialysis being centrifuged to remove suspended matter and the solution freeze-dried. The pig pancreas extract used was freeze-dried. Enzyme extracts were prepared for the experiments by grinding the enzyme powders with water and centrifuging.

The chemicals used were all crystalline laboratory grade reagents except the sodium hydrosulphite, which was a technical grade. The hide powder was from Baird and Tatlock, and the silk fibroin and the fibrin were from Hoffmann La Roche.

(c) *Measurement of Digestion*

Samples 1.00 g. of scoured dry wool were weighed and transferred to thick-walled 50 ml. centrifuge tubes containing 30 ml. of the test solution, previously adjusted to the desired pH with the aid of a glass electrode pH assembly, and stirred carefully to remove entrained air and distribute the wool uniformly through the solution. The tubes were incubated without agitation for 18 hr. at 50°C. If the wool was not broken into fragments during incubation, it was removed from the tube, washed, and squeezed repeatedly in running water. If disintegrated, the residue was filtered on a 7 cm. No. 541 Whatman filter paper on a Buchner funnel and washed well. If extensively degraded and partly gelatinized, the residue was recovered by centrifuging before suspending in water and filtering. The undigested material was dried for 5 hr. at 105°C. and allowed to equilibrate with the laboratory atmosphere overnight before weighing and calculating the percentage digestion.

TABLE 1

WOOL DIGESTION BY COMPONENTS OF PAPAIN-BISULPHITE-UREA MIXTURE ON WOOL AT 50°C., SINGLY AND IN COMBINATION

Papain at 1% Concentration	Sodium Bisulphite at 0.1M Concentration	Urea at 4.0M Concentration	Wool Digestion (%)	
			After 18 Hr.	After 41 Hr.
–	–	+	8	3
–	+	–	3	2
+	–	–	2	Nil
–	+	+	5	5
+	–	+	4	3
+	+	–	18	45
+	+	+	92	100

Samples of the standard wool, heated for 5 hr. at 105°C. and then allowed to equilibrate as above, were found to be 2 per cent. lighter than unheated equilibrated samples, and estimation of the moisture content of air-dried wool over several months in this laboratory revealed a maximum variation of ± 1.5 per cent. The small error due to the effect of heating on moisture uptake by wool and variation in weight due to changes in the relative humidity would not affect the deductions drawn in this paper from comparison of the results of separate experiments, and moreover, most comparisons are made between results obtained in the same experiment. In a series of eight tests in one digestion experiment the mean percentage digestion and the standard deviation of the mean were 43.9 ± 0.4.

III. Experimental

(a) *Digestion of Wool by Papain in the Presence of Sodium Bisulphite and Urea*

A solution containing 1 per cent. papain, 0.1M sodium bisulphite, and 4.0M urea almost completely digested 1 g. wool after 18 hr. at 50°C. The superiority of this mixture to any of the components when tested singly or in various combinations is shown in Table 1. The small amount of undigested material was ribbon-like in appearance under the microscope and was identified by Dr. E. H. Mercer as cuticular sheaths (Mercer, Lindberg, and Philip 1949). Digestion in 4M urea and 0.1M sodium bisulphite at all pH values between 5.7 and

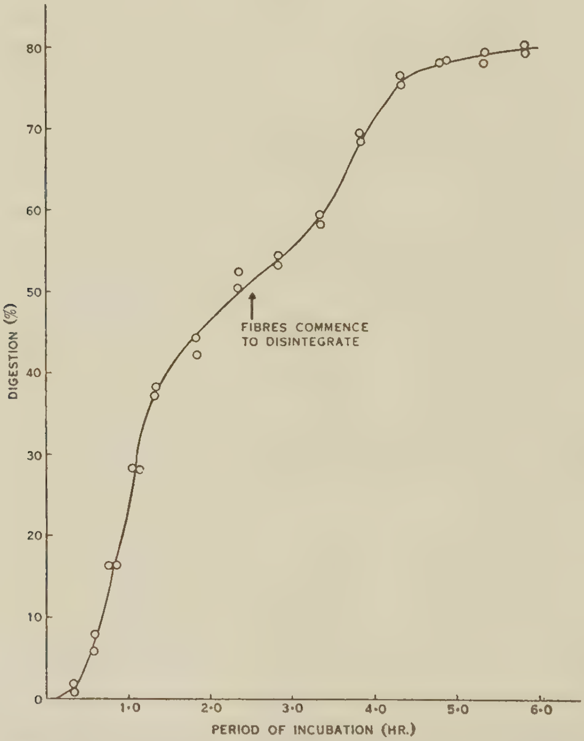

Fig. 1.—Progress of wool digestion in 1 per cent. papain, 0.1M sodium bisulphite, 3M urea during incubation at 50°C. and pH 7.0.

9.6 was inferior to that reported in the table for the papain solutions. All the amino acids of wool were detected on paper chromatography of the digest, but the separation of a white precipitate from the digest on dialysing or on adjusting the pH to 5.0-5.5, showed that portion of the wool protein was only partly degraded. This material is probably similar to that recovered by Blackburn (1950) from papain-bisulphite partial digests of wool.

The progress of digestion is shown in Figure 1. A second phase of rapid digestion followed disintegration of the fibres into fragments and cortical cells. From Figure 2, the digestion during 7 hr. incubation is seen to improve rapidly with increase in temperature above 22°C. Digestion was completed after 7 hr. at 70°C., but at 100°C. the papain was found to be inactive within 2 hr. Digestion at this temperature did not exceed 28 per cent.

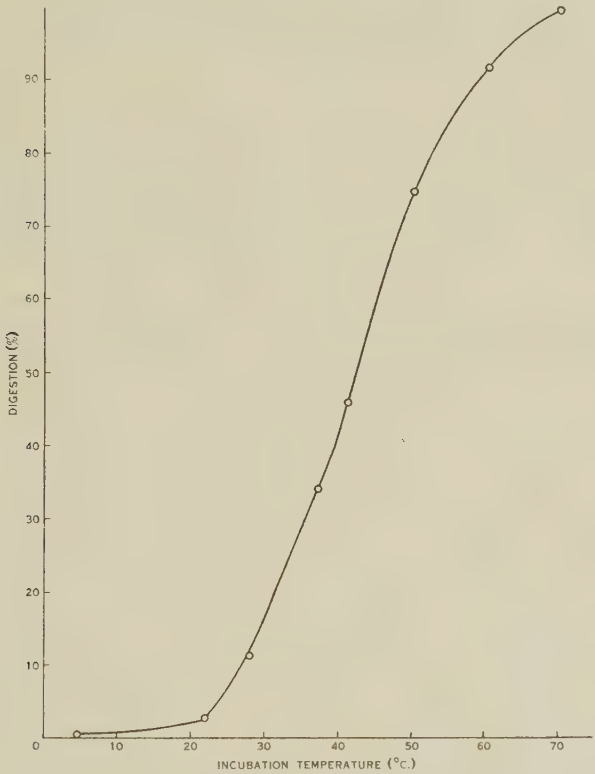

Fig. 2.—Digestion of wool in 1 per cent. papain, 0.1M sodium bisulphite, 3M urea at pH 7.0 after incubation for 7 hr. at various temperatures.

Reduction in the initial concentration of either the sodium bisulphite or urea reduced the percentage digestion during 18 hr. at 50°C., as shown in Figure 3, but it was necessary to reduce the initial papain concentration below 0.2 per cent. to produce a similar effect. Simultaneous dilution of all three components reduced the digestion much more sharply than dilution of any single component. When digested in papain-bisulphite in the absence of urea, the wool fibres were considerably weakened after 17 hr., and disintegrated into cortical cells after 41 hr.

By increasing the concentration of urea to 8M, 90 per cent. digestion occurred in 4 hr. at 50°C. (Fig. 4). With 5M urea 90 per cent. digestion

occurred in 4 hr. at 70°C., but the digestion was slightly less pronounced at higher urea concentrations at 70°C., suggesting that the enzyme itself was being inactivated at the higher urea concentrations.

By maintaining the urea concentration at 2M and increasing the sodium bisulphite concentration to 0.2M, complete digestion of the wool occurred in 2 hr. at 70°C., but at 50°C. only 40 per cent. digestion occurred during the same period and, even in 0.5M sodium bisulphite, digestion did not exceed 50 per cent. Solutions containing high bisulphite concentrations, like those containing high urea concentrations, probably inactivated the enzyme during incubation at these high temperatures.

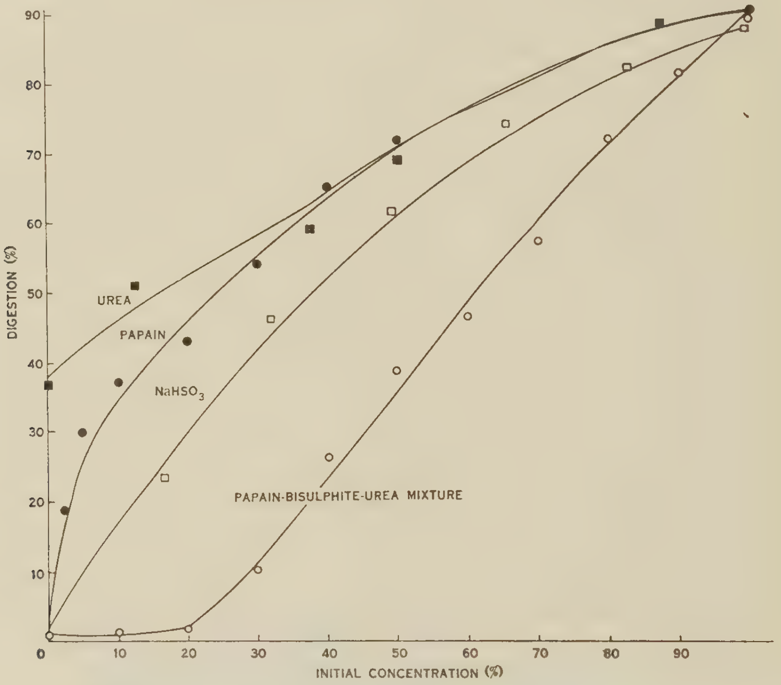

Fig. 3.—Effect of dilution of each constituent of the papain-bisulphite-urea solution on wool digestion after 18 hr. at 50°C. and at pH 7.0.
○ 2 per cent. papain-0.1M $NaHSO_3$-4M urea mixture diluted.
● 0.2 per cent. papain diluted in presence of 0.1M $NaHSO_3$ and 4M urea,
□ 0.1M $NaHSO_3$ diluted in presence of 2 per cent. papain and 4M urea,
■ 4M urea diluted in presence of 2 per cent. papain and 0.1M $NaHSO_3$.

(*b*) *Effect of pH on Digestion at Different Urea Concentrations*

The pH of a series of papain-bisulphite-urea solutions was adjusted to values between 4 and 10 with hydrochloric acid and sodium hydroxide before diluting them to 30 ml. and immersing the 1 g. wool samples. The final concentrations of the papain and the sodium bisulphite in all the solutions were

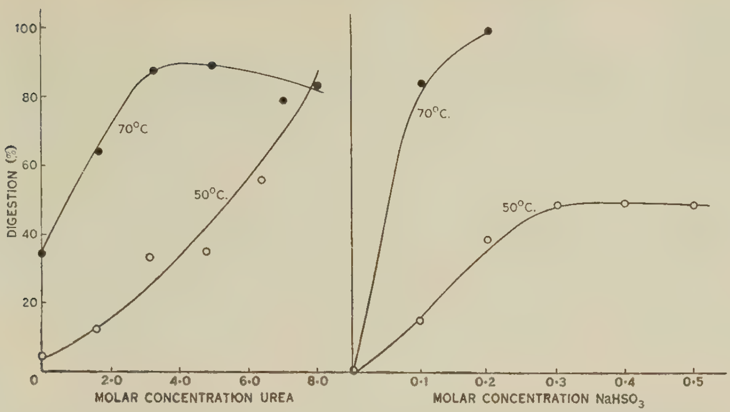

Fig. 4.—Effect of high concentrations of urea and bisulphite on digestion of wool by 1 per cent. papain after incubation for 4 hr. at 50°C. and 70°C. 0.1M $NaHSO_3$ was present in the urea series and 2M urea was present in the bisulphite series.

1 per cent. and 0.1M, respectively, but the urea was tested at different concentrations up to 4M. The pH values for maximum digestion decreased from 7.3

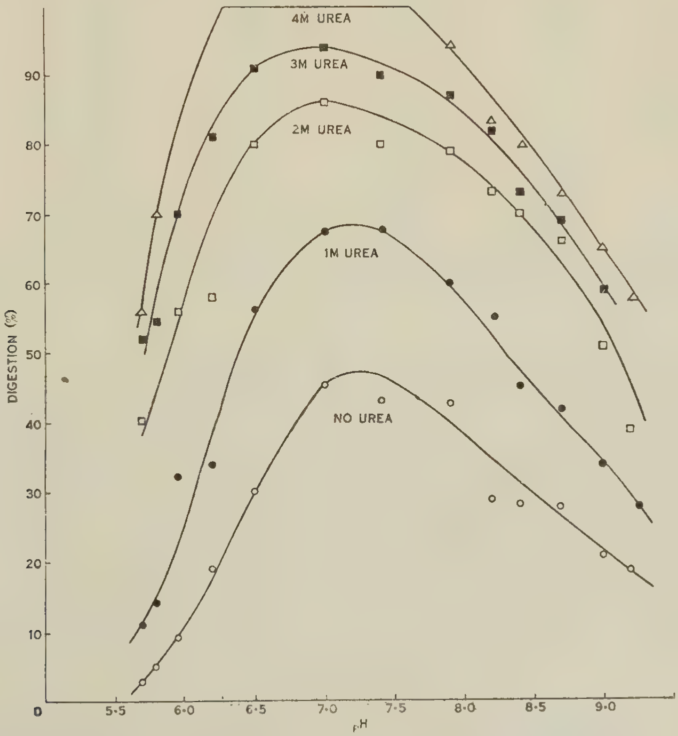

Fig. 5.—pH curves for solutions containing 1 per cent. papain, 0.1M $NaHSO_3$, and urea at the concentration indicated on each curve. The pH values reported were measured after 0.5 hr. and the digestions after 18 hr. incubation at 50°C.

to 7.0 with increase in the concentration of urea (Fig. 5). These pH values refer to readings obtained with the aid of the Beckman high temperature glass electrode 0.5 hr. after the commencement of incubation. As shown in Table 2, the pH values changed considerably immediately after the commencement of incubation, and to a lesser extent for some hours subsequently. When either papain or wool was omitted from the system, similar pH changes were observed and they are not therefore solely attributable to the uptake of ions on protein. Since wool digestion did not become properly established until after incubation for about 0.25 hr. (Fig. 1), the pH after 0.5 hr. probably approximates more closely to the mean pH for the digestion than any other.

Table 2

CHANGE IN pH OF 30 ML. 1 PER CENT. PAPAIN, 0.1M $NaHSO_3$, 3M UREA SOLUTION AFTER ADDITION OF 1 G. OF WOOL AND INCUBATION AT 50°C.

pH Before Immersing Wool	pH After the Following Incubation Periods (hr.)				
	0.01	0.17	0.50	1.50	5.50
4.0	5.0	5.2	5.7	5.7	5.9
4.5	5.2	5.4	5.8	5.8	5.9
5.0	5.5	5.7	5.9	5.8	6.0
5.5	5.9	6.1	6.2	6.1	6.2
6.0	6.4	6.5	6.5	6.4	6.5
6.5	6.9	7.0	7.0	6.9	6.9
7.0	7.3	7.4	7.4	7.4	7.2
7.5	7.9	7.9	7.9	7.8	7.5
8.0	8.2	8.1	8.2	8.0	7.8
8.5	8.5	8.4	8.4	8.3	8.1
9.0	8.8	8.7	8.7	8.5	8.3
9.5	9.1	9.1	9.0	8.8	8.5
10.0	9.5	9.3	9.2	9.0	8.8
10.5	9.9	9.7	9.6	9.4	9.1

In the following experiment the reduction of wool digestion with departure of the pH from the optimum value was shown not to be due to inactivation of the papain. A series of solutions containing 1 per cent. papain, 0.1M sodium bisulphite, and 3M urea were adjusted to the same pH values as those reported in Figure 5, and then incubated for 18 hr. at 50°C. without wool. After incubation the pH values were all adjusted to pH 6.5 and the concentrations of the constituents were reduced by dilution to 0.9 of their initial values before adding 0.1 g. wool to 3 ml. of each and incubating for 18 hr. at 50°C. The digestion varied only between 80 and 85 per cent. between the initial pH values of 4.0 and 7.0 and decreased to 72 per cent. as the pH approached 10.5.

(c) *Effect of pH on Uptake of Papain from Urea Solution*

Solutions of 0.2 per cent. papain and 3M urea were adjusted to the same pH values as in the digestion experiment. After incubating 1 g. wool in each for 1 hr. at 50°C., the wool was removed, squeezed between filter papers to

remove free liquid, transferred to solutions containing only 0.1M sodium bisulphite and 3M urea at pH 6.5, and incubated for 18 hr. at 50°C. Figure 6 shows that the digestion, and presumably also uptake of papain, were optimal at pH 7.0, there being practically no uptake of enzyme at pH 5.7.

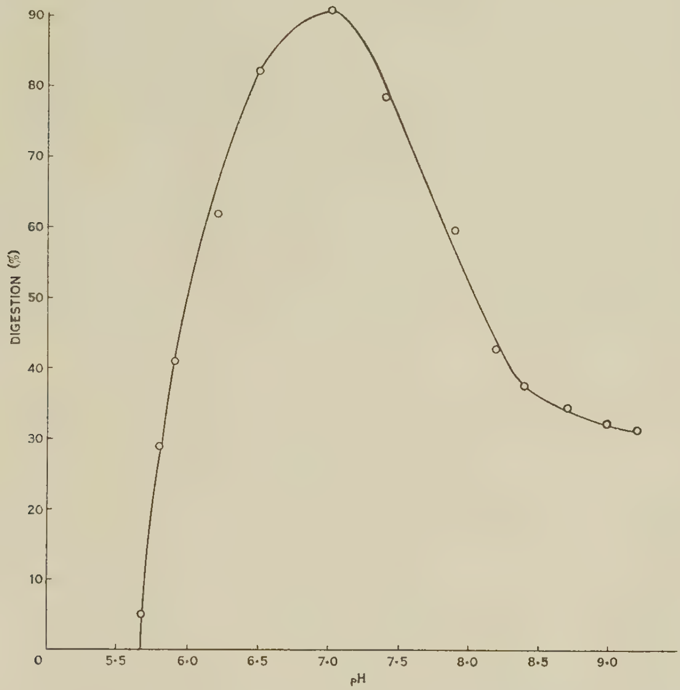

Fig. 6.—Influence of pH on uptake of papain by wool, as evidenced by digestion after 18 hr. at 50°C. in 0.1M $NaHSO_3$-3M urea, following uptake from 0.2 per cent. papain-3M urea solutions at various pH values. The pH values were measured after 0.5 hr. incubation.

In order to determine whether the uptake of papain on wool is reversed by lowering the pH, a series of papain solutions with concentrations ranging up to 1 per cent. were adjusted to pH 6.5 and 1 g. samples of wool were immersed in 30 ml. of each for 1 hr. at 50°C. The wool samples were removed from the solutions, free liquid was removed by squeezing between filter papers, they were washed by immersion in 30 ml. water for 1 hr. at 50°C., again partially dried, and transferred to 20 ml. water maintained at pH 4 by the addition of hydrochloric acid. Urea and bisulphite were added to 15 ml. of these extracts to give concentrations of 3M and 0.1M respectively after adjusting the pH to 6.5 and the volume to 30 ml.; 1 g. samples of wool were added to test the activity of these solutions by incubation for 18 hr. at 50°C. The original wool samples were partially dried and transferred to solutions containing 0.1M sodium bisulphite and 3M urea for detection of unextracted enzyme. Both sets of results are shown in Figure 7.

Although the two curves are not directly comparable, since only 20 ml. water was used for extraction and, of this, 15 ml. was taken for digestion, it is evident that extraction of the bound enzyme under these conditions was not complete.

(d) *Effect of Urea and Salts on Papain Uptake and Wool Digestion*

In the following experiment it is shown that urea influences the uptake of papain on wool. Incubation of 1 g. samples of wool for 1 hr. at 50°C. in 0.2 per cent. papain solutions at pH 6.5 containing nil, 1.0M, 2.0M, 3.0M, and 4.0M urea, followed by squeezing between filter papers and digestion for 18 hr. at 50°C. in 0.1M sodium bisulphite and 3M urea at pH 6.5, revealed an increase in wool digestion from 27 per cent. in the absence of urea to 53 per cent., corresponding to the increase in urea concentration. When urea was omitted from the bisulphite solutions, only 17-19 per cent. digestion occurred, showing that the improvement observed after transfer from papain-urea to 18 hr. bisulphite-urea digest solutions was due, not to urea taken up by the wool, but to papain uptake.

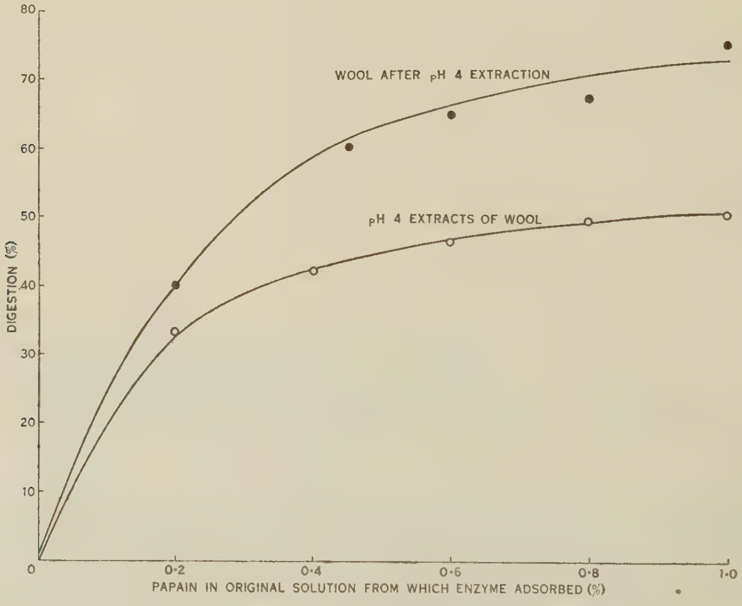

Fig. 7.—Partial removal of papain from wool at pH 4 after uptake at pH 7.0. Papain removed from wool estimated by adding 0.1M $NaHSO_3$ and 3M urea to the pH 4 extract and measuring digestion of wool after 18 hr. at 50°C. and pH 7.0. Papain not removed estimated by incubating extracted wool in 0.1M $NaHSO_3$ and 3M urea and measuring digestion after 18 hr. at 50°C. and pH 7.0.

In a similar experiment, to test the effect of sodium bisulphite on the uptake of papain on wool, the wool samples were immersed for 1 hr. in solutions at pH 6.5 containing 0.2 per cent. papain and sodium bisulphite ranging from 0.05M to 0.4M, before squeezing the samples and digesting them for

18 hr. in 0.1M sodium bisulphite and 3.0M urea at pH 6.5. The increase in bisulphite concentration was found to reduce the uptake of papain, as evidenced by a progressive reduction in wool digestion from 52 to 30 per cent. through the series. Omission of bisulphite from the 18 hr. urea digest solutions reduced the level of digestion throughout, but it revealed a progressive increase from 10 to 21 per cent. through the series. This increase was probably due to the uptake of bisulphite by the wool from the papain-bisulphite solutions.

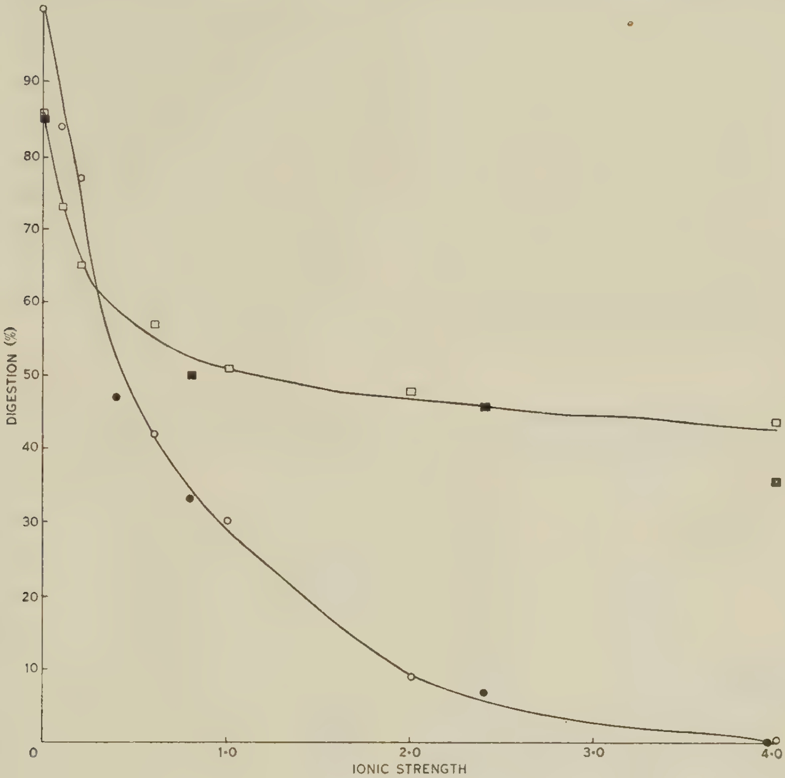

Fig. 8.—Inhibiting action of salts on wool digestion and papain uptake by wool at 50°C. and pH 7.0.

○ Digestion of wool in 1 per cent. papain, 0.1M $NaHSO_3$, 3M urea containing NaCl,

● Digestion of wool in 1 per cent. papain, 0.1M $NaHSO_3$, 3M urea containing $MgSO_4$,

□ Digestion of wool in 0.1M $NaHSO_3$, 3M urea, after adsorption of papain in the presence of NaCl,

■ Digestion of wool in 0.1M $NaHSO_3$, 3M urea, after adsorption of papain in the presence of $MgSO_4$.

In all tests digestion was measured after 18 hr. at 50°C. and pH 7.0.

The amount of wool digestion was found to be lowered by dissolving in the solution containing 1 per cent. papain, 0.1M sodium bisulphite, and 3.0M urea, 0.1M, 0.2M, 0.6M, 1.0M, 2.0M, and 4.0M sodium chloride in one experi-

ment, and 0.1M, 0.2M, 0.6M, and 1.0M magnesium sulphate in another experiment, before immersing the 1 g. samples of wool in the solutions and incubating. As shown in Figure 8, increase in salt concentration reduced the wool digestion to zero. When the concentrations were expressed in terms of ionic strength, the effects of the two salts were indistinguishable.

To study the mechanism by which wool digestion was adversely affected by salts, 1 g. samples of wool were incubated for 1 hr. at 50°C. in 30 ml. 1 per cent. papain solutions at pH 6.5, containing the same concentrations of sodium chloride and magnesium sulphate as above, then squeezed to remove excess solution, and incubated for 17 hr. at 50°C. in 0.1M sodium bisulphite, 3M urea at pH 6.5 (Fig. 8). Since the wool digestion was not inhibited to the same extent as on direct addition of salts to the papain-bisulphite-urea solution, interference with papain uptake on wool is probably not the main mechanism of inhibition. However, the possibility should be recognized that urea or bisulphite may so affect papain or wool as to render their combination more susceptible to interference by salts than this experiment suggests. The inhibition of wool digestion observed in the latter experiment may have been due to the transfer of salts on the wool to the urea-bisulphite solution.

Table 3

PERCENTAGE WOOL DIGESTION BY VARIOUS PROTEINASES WITH AND WITHOUT BISULPHITE AND UREA AT pH 7.0 AFTER 20 HR. AT 50°C.

Proteinase	1% Proteinase	1% Proteinase + 0.1M Sodium Bisulphite	1% Proteinase + 4M Urea	1% Proteinase + 0.1M Sodium Bisulphite + 4M Urea
Pepsin	Nil	Nil	Nil	6
Mould protease	Nil	5	Nil	10
Trypsin	Nil	Nil	Nil	5
Papain	Nil	30	Nil	100
Ficin	Nil	10	Nil	65
Bromelin	Nil	5	Nil	25
Beef liver cathepsin	Nil	Nil	Nil	3
Pig pancreas extract	Nil	Nil	Nil	2

(e) *Digestion Experiments with other Proteinases*

The plant proteinases ficin and bromelin partially digested wool during incubation at 50°C. in the presence of bisulphite and urea, but the other proteinases examined were ineffective at pH 7 (Table 3). The pH value-wool digestion relationships were determined for all the enzymes listed in Table 3 in the presence of 0.1M sodium bisulphite and 3M urea between pH 5.7 and pH 9.6, and for pepsin the range was extended down to pH 1. No greater digestion than that shown in Table 3 was revealed at other pH values for any proteinase except pepsin, which produced a maximum of 57 per cent.

digestion at pH 2.9 (Fig. 9).* In the absence of urea it produced maximum digestion of 48 per cent. at pH 1.8, showing that urea probably inactivated the pepsin rapidly in the previous experiment as the pH value was reduced below pH 2.9. Appreciable digestion at pH 1.8 in the absence of urea also suggests that hydrogen ions replaced urea as the denaturing agent at low pH values. In solutions containing sodium bisulphite and urea but no enzyme, maximum digestion of 15 per cent. was observed at pH 3.6. In solutions containing pepsin and urea but no bisulphite a maximum digestion of 7 per cent. was observed at the same pH value.

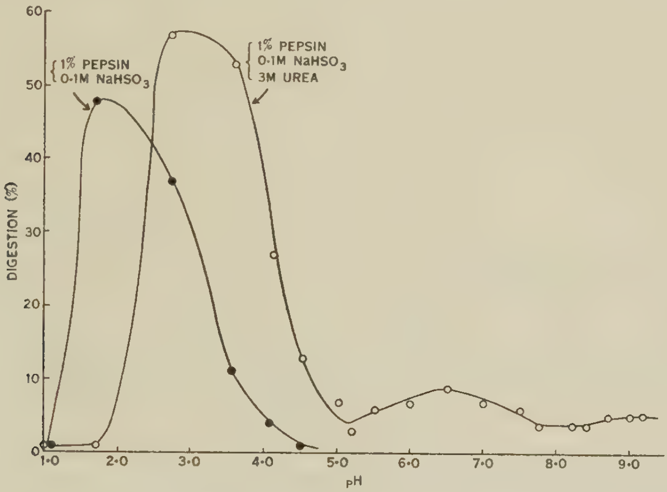

Fig. 9.—Curves relating wool digestion, after 18 hr. incubation at 50°C., with pH, after 0.5 hr. incubation at 50°C. in pepsin-bisulphite solutions with and without urea.

Incubation of wool for 1 hr. at 50°C. in 1 per cent. pepsin, 3M urea solutions, adjusted to cover a range of pH values, followed by squeezing to remove excess solution, transfer to solutions containing 0.1M sodium bisulphite and 3M urea at pH 3.0, and incubation for 18 hr. at 50°C., revealed maximum digestion of 7 per cent. corresponding to maximum uptake of pepsin at pH 3.0. This agrees fairly well with the pH of maximum digestion, but the uptake of pepsin was poor compared with the uptake of papain at the pH of maximum activity.

The effect of pH on digestion by ficin in the presence of 0.1M sodium bisulphite and 3M urea resembled its effect on papain except that the activity fell more sharply above pH 7.8 (Fig. 10), possibly owing to more rapid inactivation of the enzyme in alkaline solution.

Increasing the urea concentration up to 7.0M improved wool digestion by all the proteinases examined except ficin, which was apparently inactivated

* As in the experiment with papain, the pH values reported for the pepsin and ficin experiments refer to observations made 0.5 hr. after adding wool to the digestion mixture, that is, 0.5 hr. after the commencement of incubation.

by urea concentrations in excess of 5.0M (Fig. 11). Trypsin, beef liver cathepsin, and pig pancreas extract were the least effective, but the digestion curves for these proteinases and for pepsin rose more sharply at the high urea concentrations than at low concentrations.

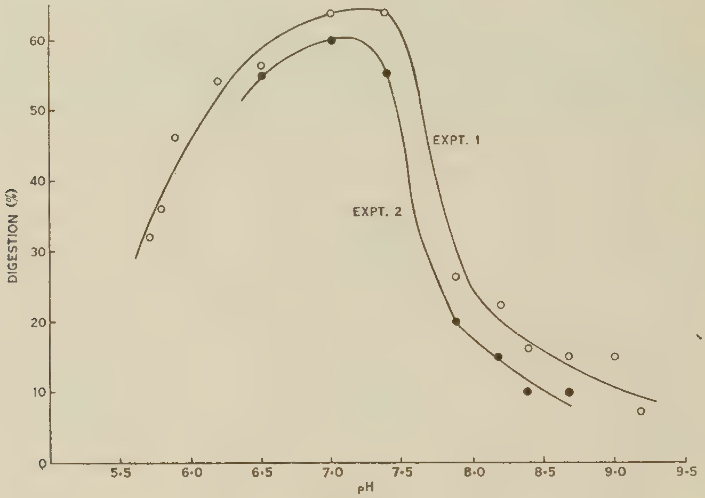

Fig. 10.—Curves relating wool digestion after incubation for 20 hr. at 50°C. in 1 per cent. ficin, 0.1M $NaHSO_3$, and 3M urea, with pH after 0.5 hr. digestion.

Although wool was completely digested in papain-bisulphite-urea, the digestion in pepsin-bisulphite-urea and ficin-bisulphite-urea did not exceed 57 per cent. and 64 per cent. respectively, even at optimal pH. Pepsin and ficin

Table 4

WOOL DIGESTION AFTER 18 HR. AT 50°C. IN PEPSIN AND FICIN SOLUTIONS CONTAINING HIGHER CONCENTRATIONS OF THE VARIOUS CONSTITUENTS THAN THOSE NORMALLY USED

Enzyme Concentration (%)	$NaHSO_3$ Concentration (M)	Urea Concentration (M)	Digestion (%) Pepsin at pH 2.7	Ficin at pH 7.0
1	0.1	3	49	41
2	0.1	3	53	40
3	0.1	3	58	41
1	0.2	3	51	49
1	0.3	3	56	57
1	0.1	5	5	56
1	0.1	7	6	66
1	0.1	3	59*	58*

* The incubation period at 50°C. was increased from 18 to 41 hr. in these experiments.

were therefore re-tested at their optimal pH, but at the higher concentrations, also in the presence of higher concentrations of sodium bisulphite and urea and with a longer incubation period, in an unsuccessful attempt to digest the wool

completely (Table 4). The pepsin was inactivated at urea concentrations greater than 3M at pH 2.7 but the ficin was unaffected. The discrepancy between the results for ficin in Table 4 and those reported elsewhere in this paper for ficin at high urea concentrations is attributable to the fact that a purchased preparation of ficin, which displays greater resistance to urea inactivation, was used only in the experiments reported in Tables 4 and 5.

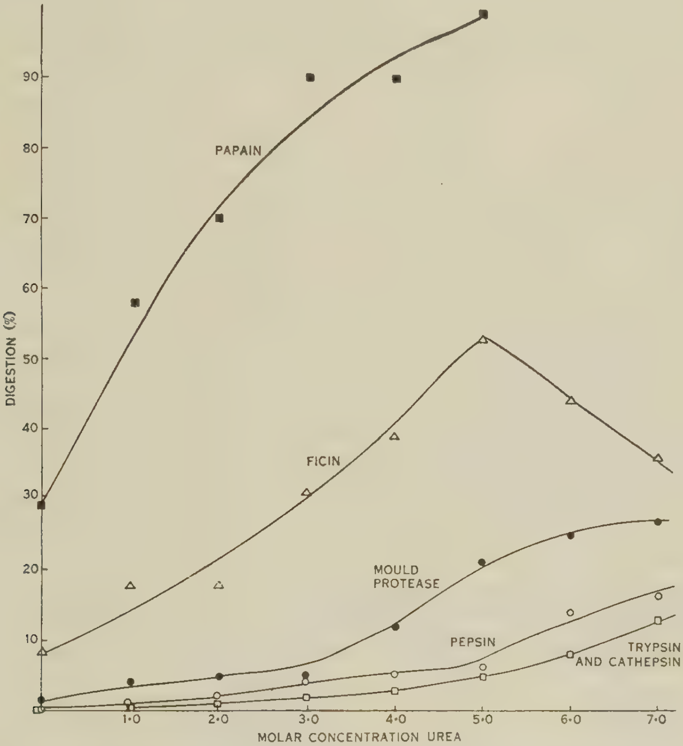

Fig. 11.—Effect of urea concentration on wool digestion during 10 hr. at 50°C. in 1 per cent. solutions of various proteinases at pH 7.0 containing 0.1M $NaHSO_3$.

The superiority of papain to the other proteinases when tested at 50°C. was also demonstrated at lower temperatures (Table 5). It cannot, therefore, be attributed to the greater thermal stability of papain.

(f) Replacement of Sodium Bisulphite with Other Reducing Agents

Owing to the difficulty of obtaining reducing agents in the pure state and of preventing oxidation, 1.0M solutions were prepared on the assumption that the compounds used were pure, and aliquots were taken and titrated with iodine. All solutions were then diluted to give the same titration as the sodium sulphide solution, immediately before adding 3 ml. of each to the papain-urea solution, adjusting to pH 6.5, and diluting to 30 ml. for measurement of wool digestion (Table 6).

Although it does not reduce iodine, potassium cyanide was included in the experiment because it is known to activate papain and it also reacts with the disulphide bonds in wool. Evidence that pretreatment with sodium thioglycollate, to produce partial reduction of wool, did not assist subsequent

TABLE 5

WOOL DIGESTION AFTER 18 HR. INCUBATION AT, AND BELOW, 50°C. IN SOLUTIONS CONTAINING 1.0 PER CENT. OF VARIOUS PROTEINASES, 0.1M SODIUM BISULPHITE, AND 3M UREA

Enzyme	pH 0.5 hr. After Immersing Wool	Digestion (%)		
		50°C.	38°C.	32°C.
Pepsin	2.7	63	43	30
Mould protease	7.0	22	14	19
Trypsin	7.0	11	9	11
Trypsin	8.4	15	11	11
Papain	7.0	98	95	70
Ficin	7.0	62	23	23
Beef liver cathepsin	7.0	21	17	10

digestion by papain-urea appreciably was obtained by immersion of wool for 2 hr. at 50°C. in 0.2M sodium thioglycollate at pH 4.6, and at pH 6.5, then washing it in running tap water and finally transferring it to solutions containing 0.2 per cent. papain and 3M urea at pH 6.5; the wool digestions ob-

TABLE 6

WOOL DIGESTION BY SOLUTIONS CONTAINING REDUCING AGENTS AT CONCENTRATIONS OF EQUIVALENT IODINE TITRATION, IN PRESENCE OF 1 PER CENT. PAPAIN AND 3M UREA AT pH 7.0 AFTER 18 HR. AT 50°C.

Compound	Each 1 ml. Reagent Solution Diluted to Following Volume to Equal Iodine Titration of Na_2S Solution (ml.)	Digestion (%)
Sodium bisulphite	3.73	38
Sodium hydrosulphite	3.07	32
Sodium sulphide	1.00	31
Cysteine hydrochloride	1.96	28
Sodium thioglycollate	1.77	24
Potassium cyanide	—*	7

* Not oxidized by iodine but tested without dilution.

served after 18 hr. at 50°C. were 6 and 26 per cent. respectively, which are no greater than occurred in the presence of papain-thioglycollate-urea at the same pH. In other instances the reduced wool was transferred to papain-urea solutions containing also 0.1M potassium cyanide, which is known to activate papain but does not confer wool-digesting action on papain-urea solutions at

pH 6.5. This modification caused the wool digestion to increase to 33 per cent. Thioglycollate reduction in solutions adjusted to pH 6.5 was shown to predispose the wool to digestion more than reduction at pH 4.6, but cyanide effected little further improvement. Thus reduction of the disulphide bonds in wool by thioglycollate in acid solution was less effective than sodium bisulphite reduction, judging from digestibility in papain at pH 7.0.

Although the data presented in this paper suggest that bisulphite is more effective than other reducing agents in assisting the digestive action of papain-urea solutions on wool, some reducing agents are more effective than bisulphite in alkaline solution. Experiments describing these observations will be reported in another paper.

(g) *Replacement of Urea with Related Compounds*

Replacement of the urea in the papain-bisulphite-urea solution with closely related compounds showed that they too assisted digestion. Unrelated compounds, however, such as potassium thiocyanate and potassium trichloracetate, which were selected for test on the basis of their exceptional activity in lowering the shrinkage temperature of collagen (Lennox 1949), were only slightly effective. They were tested at concentrations ranging from 0.5M to 3.5M at pH 7.0 in the presence of 2 per cent. papain and 0.1M sodium bisulphite, but even after 3 days' incubation at 50°C. digestion at the most favourable concentrations, namely 1.0M potassium thiocyanate and 0.5M potassium trichloracetate, did not exceed 60 per cent. Urea at 3.5M concentration, on the other hand, caused complete digestion in 18 hr.

Substitution of various compounds for urea in the 1 per cent. papain, 0.1M sodium bisulphite, urea solution yielded the following wool digestions during 18 hr. at 50°C. at pH 7:

4M and 10M ethanol	19 per cent. digestion
4M semicarbazide hydrochloride	4 per cent. digestion
1.7M sodium salicylate	1 per cent. digestion.

The following compounds produced no digestion:

4M potassium thiocyanate, 4M ammonium chloride, 4M glycine, 4M hexamethylenetetramine, 4M alloxan, 10M lithium chloride, 0.4M cetyltrimethylammonium bromide, 0.4M sodium lauryl sulphate.

Of the compounds reported in Table 5 having molecular structures closely related to that of urea, thiourea, in which the O atom of urea is replaced by a S atom, was almost equally effective at only half the concentration, but low solubility prevented its use at higher concentrations. The superiority of thiourea was not apparent in the absence of bisulphite, for the percentage digestions by 1M urea and 1M thiourea were only 9 and 8 respectively, whereas in the presence of 0.1M $NaHSO_3$ in the same experiment, they were 63 and 81 respectively. Guanidine, having the urea O replaced by NH, was next in effectiveness, and then followed ethyl urea and methyl urea in which one of the H atoms of the NH_2 group in urea was substituted. Still less effective were urethane, formamide, and acetamide, in which an NH_2 group in urea has been entirely replaced, and finally acetamidine, which bears the same

structural relationship to guanidine as acetamide does to urea. The relative activities of thiourea, urea, and guanidine hydrochloride, shown in Table 7, were confirmed in another experiment in which the thiourea and urea had been recrystallized from water and the guanidine hydrochloride had been purified by precipitation from methanol by the addition of ether, as recommended by Greenstein and Jenrette (1942).

TABLE 7

WOOL DIGESTION AFTER 18 HR. AT 50°C. IN 1 PER CENT. PAPAIN, 0.1M SODIUM BISULPHITE SOLUTIONS CONTAINING COMPOUNDS STRUCTURALLY RELATED TO UREA, AT pH 6.5

Compound	Digestion (%) Expt. 1	Expt. 2
2M Thiourea	92	90
2M Urea	69	66
4M Urea	93	96
4M Guanidine	73	74
4M Ethyl urea	68	73
4M Methyl urea	67	63
3M Urethane	54	50
4M Formamide	44	48
4M Acetamide	41	44
4M Acetamidine	19	12

(*h*) *Action of Papain-Bisulphite-Urea on Other Fibrous Proteins*

The extent of digestion of keratinous materials other than wool and of other fibrous proteins is shown in Table 8. When tested in a coarsely ground state, the digestion of horn was much less than that reported in the table. The digestion of silk fibroin was optimal at pH 6.5, but the digestion of fibrin increased to 48 per cent. at pH 7.5. In the absence of urea only 5 per cent. of the fibrin was digested.

IV. DISCUSSION

The favourable effect of urea on the digestion of wool keratin by papain-bisulphite is probably related to its well-known "denaturing" and solvent effects on globular proteins. Anson and Mirsky (1929) reported such action on haemoglobin, ovalbumin, and serum albumin, and Hopkins (1934) studied the action of urea on crystalline ovalbumin and observed that the effect was shared, though to a lesser extent, by many other compounds related to urea. He concluded that substitution of more than one of the two NH_2 groups in the urea molecule greatly reduced the activity. Burk and Greenberg (1930) observed that urea causes pronounced disaggregation of haemoglobin and edestin into units of lower molecular weight.

It seems likely that urea acts by competing with various groups of the protein for their place in hydrogen bonds, thereby reducing the cohesion of the structure. A reduction in the work of extension of wool and hair in the

presence of 5M urea was reported by Pasynskii and Blokhina (1950). Similarly, weakening of keratin by sodium bisulphite was shown by Carter, Middlebrook, and Phillips (1946) to occur under conditions which lead to the conversion of about half of the disulphide bonds contributed by the cystine residues to thiol and S-cysteine sulphonate groups. The bisulphite and urea, in conjunction with hydrolysis of peptide bonds by the papain, presumably disrupt the keratin sufficiently for the fragments to pass into solution.

TABLE 8

DIGESTION IN SOLUTION CONTAINING 1 PER CENT. PAPAIN, 0.1M SODIUM BISULPHITE, AND 3M UREA AT pH 7.0 OF KERATINOUS AND OTHER FIBROUS PROTEIN MATERIALS AFTER 18 HR. AT 50°C.

Protein Material	Digestion (%)
Wool	90
Cattle horn filings	96
Duck feathers, finely ground	87
Hide powder	100
Sheepskin, shorn	100
Fibrin	25
Silk	22
Silk fibroin	26
Nylon	Nil

The possibility that urea favoured papain digestion by increasing the dielectric constant of the medium is untenable since glycine, which greatly increases the dielectric constant, did not affect digestion. Urea may so affect the outer layers of wool as to facilitate their digestion and thereby allow ingress of enzyme and egress of soluble proteins, but apparently it also facilitates direct attack on the cortical cells, for when liberated after 2 days' incubation of wool in papain-bisulphite solution, they are digested less rapidly in the absence of urea than are the intact fibres.

Although bisulphite and urea both facilitate digestion of wool by papain, the observed differences in the rate of digestion of wool by various proteinases are partly attributable to differences in the degree of inactivation of the enzyme by urea or bisulphite. In general, those enzymes which are activated by reducing agents are stable to bisulphite; so also is pepsin. Trypsin, on the other hand, is inactivated by reducing agents (Grob 1946), and W. G. Crewther (unpublished data) has demonstrated partial inactivation of mould protease by sodium sulphite at concentrations corresponding to the bisulphite concentrations used in the present investigation. Many enzymes are known to resist high concentrations of urea. Steinhardt (1938), for example, showed that pepsin digests proteins in the presence of 4M urea, and in the Anson (1938) method of estimating trypsin and papain activity, urea is added to the solution to denature the haemoglobin substrate. Lineweaver and Schwimmer (1941) showed that papain retains its capacity for hydrolysing casein even

after incubation for 24 hr. at 30°C. in 9M urea. Of all the proteases examined, papain, which is activated by reducing agents and is also remarkably stable to denaturing influences such as heat and urea, produced the greatest digestion. The failure of ficin and pepsin to produce complete digestion of wool, even in the presence of three times the normal concentration of enzyme or bisulphite, more than twice the normal concentration of urea, or at temperatures below 50°C., or during prolonged incubation, showed that papain possessed some property, apart from pronounced resistance to high temperatures, bisulphite, and urea, that enabled it to digest rapidly all the components of wool. By virtue of their size or shape, papain molecules may be able to penetrate more readily than molecules of the other proteases to particular peptide bonds that are essential to the molecular structure of wool fibres. Alternatively, the more resistant components of wool may satisfy the particular substrate requirements of papain, but not of the other proteinases examined.

The action of urea on wool, in contrast with the effect of certain anions previously shown to be highly effective in lowering the thermal stability of collagen (Lennox 1949), suggests that the stability of the molecular network of keratin is less dependent on salt linkages than on hydrogen bonds and disulphide bonds, whereas collagen is stabilized mainly by salt linkages.

Elsworth and Phillips (1938) showed that the breakdown of disulphide bonds of wool in bisulphite solutions proceeded optimally at pH 5. The contribution of this reaction to the digestion of wool keratin is apparently less important than the uptake of papain which, like wool digestion, was most pronounced at pH 7. This optimal value is close to that reported by Middlebrook and Phillips (1941).

Titration curves for wool indicate that the isoelectric point of wool keratin is approximately pH 6.2 (Steinhardt and Harris 1940; Lemin and Vickerstaff 1946), whereas that for papain is approximately pH 9.0 (Balls and Lineweaver 1939). It is perhaps significant that the pH of optimal uptake of papain on wool and wool digestion lies between the isoelectric points of the enzyme and its substrate, for under these conditions they would be oppositely charged. Also, since the fibrous nature of the wool keratin would prevent folding and internal neutralization of charged groups of the type occurring in globular proteins near the isoelectric point, isoelectric keratin would be highly receptive for other ionized compounds such as papain. Northrop, Kunitz, and Herriott (1948) have similarly suggested that the pH optima for the digestion of proteins by pepsin and trypsin partly depend on combination of these enzymes with positively charged and negatively charged protein substrate ions respectively.

V. Acknowledgments

The author's thanks are due to Mr. J. M. Gillespie for preparing the freeze-dried mould enzyme and pig pancreas enzyme preparations used in this work, and to Mrs. H. M. Forss for her very careful technical assistance.

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NITROGEN CATABOLISM IN NEMATODE PARASITES

By W. P. Rogers*

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Summary

Nematodirus filicollis, Nematodirus spathiger, and *Ascaridia galli* were maintained for periods of 24 hours in a non-nutrient medium containing streptomycin and penicillin to prevent bacterial growth. Under aerobic conditions the total soluble nitrogenous material excreted by *Nematodirus* spp. in three experiments was 1.23-1.59 mg. nitrogen per g. dry wt. of tissue. Peptide nitrogen accounted for 33-37 per cent. of the total soluble nitrogen excreted, ammonia nitrogen 40-42 per cent., and urea nitrogen 11-17 per cent. Small amounts of uric acid were found. Under similar conditions the total soluble nitrogen excreted by *Ascaridia galli* was 0.29-0.41 mg. per g. dry wt.; of this, 14-15 per cent. was due to peptide nitrogen, and 8 per cent. to urea nitrogen. No uric acid was found.

Under anaerobic conditions, excretion of soluble nitrogenous material by *Nematodirus* spp. was increased 40-42 per cent.; *Ascaridia galli* was not appreciably affected.

Ammonia was formed in brei prepared from *Nematodirus* spp. and *Ascaridia galli*; the amounts found were increased in the presence of added urea, alanine, aspartic acid, and glutamic acid. Urea production was greatly increased by adding citrulline, ornithine, and arginine. Cobalt ions, 10^{-3}M, increased urea production in the presence of arginine. It was concluded that urea formation in the tissues of nematode parasites took place via a citrulline cycle similar to that described by Krebs and Henseleit (1932).

Urease and arginase activity in homogenates prepared from the intestine of *Ascaris lumbricoides* were very much greater than in homogenates prepared from the ovary, or muscle, which included the lateral line organs.

Of the purine derivatives examined, only muscle adenylic acid, and, to a less extent, adenine, caused increased ammonia formation in brei prepared from mature *Ascaridia galli, Nematodirus* spp., or the muscle of *Ascaris lumbricoides*. Young adult *Ascaridia galli,* from infestations 4-5 weeks old, formed large quantities of urea and ammonia from added xanthine, uric acid, and allantoin. Guanase activity was absent. It was concluded that the breakdown of purines in nematode parasites, when it occurs, takes place by the action of uricase, allantoinase, allantoicase, and urease.

I. Introduction

In nematode parasites, where the major metabolic activities are directed towards egg production, the emphasis on protein and nucleic acid synthesis must be considerable, and nitrogen metabolism generally might be expected to be rapid. The catabolic processes of nitrogen metabolism, however, appear

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to be relatively slow, and most workers agree that the products usually associated with the breakdown of protein in other animals have not been found in appreciable quantities in media in which adult parasites have been maintained (Lapage 1937). Thus the total nitrogen excreted by *Ascaris lumbricoides* in 24 hours ranges from 15 to 29 mg. per 100 g. wet weight (Weinland 1904; von Brand 1934). About one-third of the excreted nitrogen is ammonia (Weinland 1904). The remainder consists of substances precipitated by phosphotungstic acid, amino acids, small amounts of amines, and mercaptan (Flury 1912, Weinland 1904). Chitwood (1938) found a small quantity of urea in fluid collected from the excretory pore of *Ascaris* but considered that it probably came from the host. About a quarter of the excreted nitrogen is in the eggs discharged by the parasites (von Brand 1934).

There are a number of difficulties in determining the nature of the excretory metabolism of nematode parasites. The most important are those due to the inability to study the parasites under the physiological conditions in which they exist in host animals, and inability to avoid the misleading results that might be caused by the presence of bacteria in the medium. The results reported in this paper were obtained with parasites kept in non-nutrient and glucose-containing media, to which antibiotics had been added to limit the growth of bacteria. The results, therefore, apply to parasites living under abnormal conditions. However, the possible errors in earlier work, arising from bacterial contamination, have been lessened.

Most of the nematodes examined were parasites of the small intestine. They were *Ascaridia galli,* from the chicken, *Nematodirus filicollis* and *N. spathiger* from the sheep, and *Ascaris lumbricoides* from the pig. The nature of the chief nitrogenous excretory products, and some of the biological processes leading to the formation of these materials have been examined.

II. Methods

(*a*) *Biological Materials*

Nematodirus filicollis and *N. spathiger,* which were not separated for use, and *Haemonchus contortus,* were obtained from naturally infested sheep; *Ascaris lumbricoides* from naturally infested pigs, and *Ascaridia galli* from experimentally infected chickens.

The parasites were washed in saline till free of debris, and then washed several times in sterile saline if they were to be used in "culture" experiments. *Ascaridia galli* was cleaned and placed in media within 30 minutes of the death of the host; the cleaning of *Nematodirus* spp. took up to 2 hours. *Ascaris lumbricoides* was not used for "culture" experiments.

(*b*) *Culture Experiments*

The basal medium contained 8.8 g. NaCl, 0.42 g. KCl, and 0.48 g. $CaCl_2.6H_2O$ per litre. To this was added 100 ml. of 0.15M phosphate buffer at pH 6.8. When glucose medium was used, 50 ml. of isotonic glucose solu-

tion was added per litre of the basal medium. Streptomycin and penicillin, in saline, were added in various amounts to the basal medium. Solutions were sterilized by filtering through sintered-glass bacterial filters. Air was used as a gas phase for aerobic preparations; anaerobiosis was obtained by passing nitrogen, which had been freed of oxygen over copper turnings at 400°C., through the medium containing the parasites.

The "cultures" were set up in small Erlenmeyer flasks in pairs so that the results obtained in aerobic or anaerobic containers, or in the presence or absence of glucose, could be compared with parasites from the same source. The flasks were provided with stopcocks, which were closed after gassing. For *Ascaridia galli*, 8 ml. of medium was used for 10 parasites of total weight about 0.5 g. The same proportion of males and females, usually 1 to 10, was used in each pair of flasks. For *Nematodirus* spp. 250 mg. of mixed males and females were used in 10 ml. of medium.

After incubation at 37°C. for 24 hours, a sample, 0.5 ml. of the medium, was taken for bacteriological examination. The parasites were taken from the medium and washed with 2 ml. of fresh medium. The washings were added to the original medium which was then centrifuged. The supernatant solution was used for chemical analysis; the analytical results given in this paper thus refer to soluble nitrogen compounds only. The solid material, which consisted largely of eggs, was suspended in water and the eggs in suitable samples were counted. The parasites were freed of excess moisture with filter paper and weighed.

Samples for bacteriological examination were diluted and cultured on serum agar anaerobically and aerobically. *Nematodirus* spp. maintained under aerobic conditions were frequently contaminated with bacteria, even when high concentrations of antibiotic were present. Only those that were sterile, or contained less than 100 organisms per ml., were used for analysis.

Brei or mince, prepared by the method of Seevers and Shideman (1941), was brought to the appropriate pH and suspended in $^{M}/_{15}$ phosphate buffer or Krebs-Ringer phosphate without calcium chloride.

(*c*) *Chemical Methods*

Ammonia was estimated by a modification of the method of Conway and Byrne (1933). After distillation, the ammonia was estimated in a Beckman spectrophotometer with Nessler reagent at 530 mμ. Urea was estimated manometrically (Krebs and Henseleit 1932; Krebs 1942) and by the method of Conway (1933). The urease was prepared from soy beans. Uric acid was estimated colorimetrically, with and without treatment with uricase (Buchanan, Block, and Christman 1945). Owing to the presence of peptides in the media, phosphotungstic acid was used as a precipitant instead of sodium tungstate. Total nitrogen was estimated by the micro-Kjeldahl method, and protein and polypeptide nitrogen were estimated similarly after precipitation with trichloracetic acid and phosphotungstic acid (Gotfried 1939).

The L–(+)citrulline was prepared from arginine by the method of Gornall and Hunter (1939) using arginase from ox liver (Hunter and Dauphinee 1930). The other substrates were commercial products of high purity. Adenosine-triphosphate was prepared as the barium salt by the method described by Umbreit, Burris, and Stauffer (1946). It was converted to the sodium salt immediately before use.

III. Procedure and Results

(*a*) *Behaviour of the Parasites in Artificial Media*

When penicillin, about 1000 units per ml., and streptomycin, 0.4 mg. per ml., were present in the medium, the anaerobic preparations of *Nematodirus* spp. were found to be sterile, but only about 15 per cent. of the aerobic preparations were suitable for analysis. Contamination at high antibiotic concentrations was due to *Serratia* sp. The aerobic preparations of *Ascaridia galli* which contained antibiotics were usually sterile, but the anaerobic ones were frequently contaminated with *Clostridium* sp.

Before examining the nature of the nitrogen compounds excreted by the parasites, it was necessary to examine the suitability of the medium. This was done by observing the egg production, activity, and gross morphological changes of the parasites while they were kept in the medium.

For this purpose, 50 female *Nematodirus* spp. or two female *Ascaridia galli* were maintained in 10 ml. of medium, which was replaced every 24 hours. Under aerobic conditions the parasites remained active and appeared healthy for 9-14 days; under anaerobic conditions the parasites soon became inactive but became active again on exposure to air up to the fifth day. No obvious morphological changes occurred in *Ascaridia galli* but in old "cultures" the cuticle of *Nematodirus* spp. became inflated in about 30 per cent. of the parasites examined. Egg production by both parasites fell rapidly until the third day after which no eggs were produced. Under anaerobic conditions, egg production was about a half to a third of that found under aerobic conditions. The addition of glucose to the medium, or the use of bicarbonate-carbon dioxide buffers, did not appreciably affect the egg-laying capacity of the parasites. Except for the addition of glucose to the medium, no attempt was made to provide food for the parasites. It is considered that their behaviour indicated that the medium was not toxic and that it was suitable for the study of the excretion of nitrogenous products by fasting parasites for the first 24 hours.

(*b*) *The Nitrogenous Compounds Excreted*

The results of three analyses carried out with several lots of *Nematodirus* spp. and *Ascaridia galli* under aerobic and anaerobic conditions are shown in Table 1. The nitrogen in the streptomycin and penicillin was estimated, and corrections for its presence in the medium were made. *Nematodirus* spp. excreted about five times as much soluble nitrogenous material as *Ascaridia galli*. In aerobic cultures of *Nematodirus* spp., polypeptide nitrogen accounted

for 33-37 per cent. of the total nitrogen excreted; ammonia nitrogen 40-42 per cent.; urea nitrogen 11-17 per cent., and uric acid 2-4 per cent. These components accounted for about 90 per cent. of the total soluble nitrogen excreted. In aerobic cultures of *Ascaridia galli,* polypeptide nitrogen accounted for 14-15 per cent.; ammonia nitrogen 52-58 per cent., and urea nitrogen 9-18 per cent. Uric acid was not found in any of the *Ascaridia galli* "cultures." The pattern of nitrogen excretion in *Ascaridia galli* did not alter much in changing from aerobic to anaerobic conditions, though the total excretion was increased by 3-13 per cent. The nitrogen excretion of *Nematodirus* spp. was increased by 20-40 per cent. under anaerobic conditions; the polypeptide nitrogen was increased in amount though it still formed 32-39 per cent. of the total soluble nitrogen excreted. The amount and proportion of ammonia nitrogen, 27-31 per cent., and urea nitrogen, less than 8 per cent., were decreased and it appears that the increased nitrogen excretion was mainly due to unidentified material.

TABLE 1

NATURE AND AMOUNT OF SOLUBLE NITROGEN COMPOUNDS EXCRETED BY FASTING *NEMATODIRUS* SPP. AND *ASCARIDIA GALLI* IN AEROBIC AND ANAEROBIC MEDIA (MG./G. WET WT./24 HR.)

The figures between brackets show the percentage partition of the nitrogen in the excreta of the parasites

Culture	Total N	Polypeptide N	Ammonia N	Urea N	Uric Acid N	N Accounted For (%)
Nematodirus spp.						
Aerobic 1	1.29	0.43 (34)	0.54 (42)	0.20 (15)	0.06 (5)	96
Aerobic 2	1.57	0.58 (37)	0.66 (42)	0.17 (11)	0.04 (2)	92
Aerobic 3	1.23	0.41 (33)	0.49 (40)	0.21 (17)	0.11 (9)	99
Anaerobic 1	1.76	0.56 (32)	0.48 (27)	0.01 (1)	0.03 (2)	62
Anaerobic 2	1.93	0.76 (39)	0.59 (30)	0.08 (4)	0.07 (4)	78
Anaerobic 3	1.48	0.49 (33)	0.42 (29)	0.10 (7)	0.10 (7)	75
Ascaridia galli						
Aerobic 1	0.29	— —	0.18 (52)	0.04 (15)	Nil (Nil)	—
Aerobic 2	0.35	0.05 (14)	0.20 (57)	0.03 (9)	Nil (Nil)	80
Aerobic 3	0.41	0.06 (15)	0.24 (58)	Nil (Nil)	Nil (Nil)	73
Anaerobic 1	0.32	— —	0.16 (50)	0.06 (19)	Nil (Nil)	—
Anaerobic 2	0.37	0.06 (16)	0.21 (56)	0.04 (10)	Nil (Nil)	82
Anaerobic 3	0.42	0.06 (14)	0.30 (72)	Nil (Nil)	Nil (Nil)	86

(*c*) *Formation of Ammonia*

Mince prepared from *Ascaridia galli, Nematodirus* spp., and *Haemonchus contortus,* suspended in $^{M}/_{15}$ phosphate buffer at pH 7.3, was incubated at 37°C. At intervals, samples were taken for the estimation of ammonia. In different lots of *Nematodirus* spp. ammonia production ranged from 0.3 to 1.1 mg. per g. dry wt. of tissue per hour. Somewhat similar figures were obtained with *Ascaridia galli.* The one experiment carried out with *Haemonchus contortus* gave a very low ammonia production.

(*d*) *Source of the Excreted Ammonia*

The possibility that the ammonia found in the medium was the result of urease activity in the tissues of the parasite was first examined. The activity of urease was estimated manometrically at pH 5 with mince prepared from *Ascaridia galli* and *Nematodirus* spp. In the presence of added urea, carbon dioxide production was increased, but the amount was so small that it could not be measured conveniently in semi-micro Warburg vessels. Urease activity was therefore examined by measuring the ammonia formed in the presence of added substrate, in Conway microdiffusion vessels. The results of one experiment with *Nematodirus* spp. are shown in Figure 1. Urea, 2 mg., in 1 ml. $^{M}/_{15}$ phosphate buffer at pH 7.3, or phosphate buffer alone, was added to 3 ml. of a suspension of mince in Krebs-Ringer phosphate. At intervals, enzyme activity was stopped by adding glacial acetic acid and boiling, and the ammonia was determined. The addition of urea caused a large increase in ammonia production. *Ascaridia galli* showed a slightly lower urease activity than did *Nematodirus* spp.; *Haemonchus contortus* showed about one-tenth of the activity of the other species.

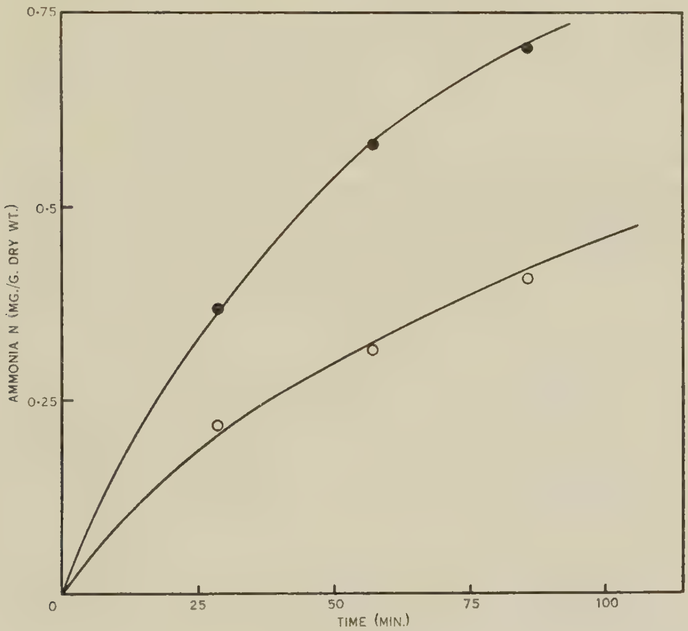

Fig. 1.—Urease activity in the tissues of *Nematodirus* spp.; ●, ammonia production in the presence of added urea; O, ammonia production in the control preparations.

Urease, of activity approaching that of the whole tissue of *Nematodirus* spp., could be extracted from brei with $^{M}/_{15}$ phosphate buffer at pH 7.5 in about 18 hours at 5°C.

(*e*) *Formation of Ammonia from Amino Acids*

The amino acids *DL*-aspartic acid, *L*-alanine, *L*-arginine, and *L*-glutamic acid, were added at pH 7.3 to brei prepared in Krebs-Ringer phosphate solution to give a final concentration of 0.01M of the active isomer. The ammonia found in experiments with *Nematodirus* spp. is shown in Table 2. In other similar experiments the increase in ammonia production due to the amino acids varied greatly, but in all instances the ammonia produced in the presence of alanine was less than that found when the other amino acids were used. Somewhat similar results were obtained with brei prepared from *Ascaridia galli.*

TABLE 2

FORMATION OF AMMONIA IN BREI OF *NEMATODIRUS* SPP. IN THE PRESENCE OF AMINO ACIDS

Amino Acid Added	Ammonia N (mg./g. dry wt./hr.)
None	0.21
Alanine	0.43
Aspartic acid	0.63
Glutamic acid	0.61
Arginine	0.64

(*f*) *Source of the Excreted Urea*

The occurrence of urea in the medium in which the parasites had been maintained, and the increased ammonia production in brei, known to contain urease, when certain amino acids were added, suggested that the ornithine cycle of Krebs and Henseleit (1932) might function in the tissues of the parasites. Arginase activity in the parasites was therefore examined.

TABLE 3

EFFECT OF CO^{++} ON ARGINASE IN *ASCARIDIA GALLI*

Concentration of Co^{++}	Urea N + Ammonia N (mg./g. dry wt./hr.)
Nil	0.61
10^{-5}M	0.63
10^{-4}M	0.79
10^{-3}M	0.83
10^{-2}M	0.77

Brei was prepared, brought to pH 9.5, and glycine buffer of pH 9.5 was added. Arginine buffered to pH 9.5 was added to a final concentration of 0.01M. All these preparations were carried out with chilled materials and vessels. The mixture was then incubated at 37°C., and at intervals, samples were taken for the estimation of ammonia and urea. One set of results ob-

tained with *Ascaridia galli* is shown in Figure 2. Somewhat similar results were obtained with *Nematodirus* spp. and *Ascaris lumbricoides* muscle. Urea production was always greatly increased in the presence of added arginine.

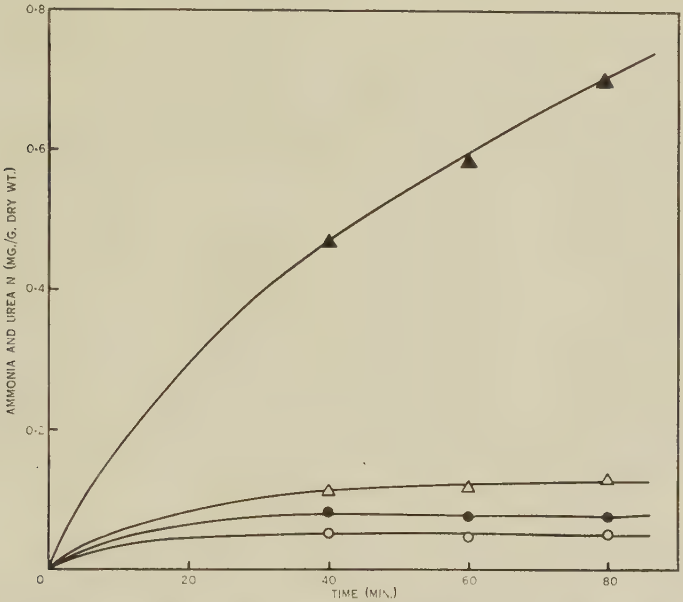

Fig. 2.—Arginase activity in the tissues of *Ascaridia galli*, ▲, urea production in the presence of added arginine; △, ammonia production in the presence of added arginine; ●, urea production in the control preparations; O, ammonia production in the control preparations.

The action of cobalt ions in activating the arginase was studied. Urea and ammonia formation was examined over the period of one hour in brei containing arginine and cobaltous chloride at concentrations of 10^{-5}M, 10^{-4}M, 10^{-3}M, and 10^{-2}M. The results obtained with *Ascaridia galli* are shown in Table 3. A concentration of cobalt ions at about 10^{-3}M caused a considerable increase in the urea and ammonia formed. Somewhat similar results were obtained with brei from *Nematodirus* spp.

The production of urea from the intermediates of the Krebs and Henseleit cycle was examined in the presence of added oxaloacetic acid. Adenosinetriphosphate was not added because it was rapidly deaminated in the brei and the high ammonia production masked the results obtained when citrulline and ornithine were added. Brei was prepared in Krebs-Ringer phosphate and freshly prepared, neutralized oxaloacetic acid, ornithine, and citrulline were added. The mixtures, in Erlenmeyer flasks, were shaken in an atmosphere of air for one hour at 37°C. after which the ammonia and urea formed were determined. The results obtained with brei prepared from *Ascaridia galli* and

the intestine of *Ascaris lumbricoides* are shown in Table 4. The increased production of urea and ammonia when ornithine and particularly when citrulline were added to the brei suggests that some form of the Krebs and Henseleit cycle was responsible for the synthesis of urea in the tissues of the parasites.

(*g*) *Distribution of Urease and Arginase in the Tissues of* Ascaris lumbricoides

Intestine, muscle, and ovary were dissected from *Ascaris lumbricoides,* washed in chilled saline, and weighed after removing excess fluid with filter paper. The materials were homogenized in a small glass mill of the type described by Potter and Elvehjem (1936) with two volumes of chilled phosphate buffer at pH 7.4. The "muscle" included a small amount of cuticle and a large part of the lateral line organs. Only the fine, convoluted tubules of the ovary were used. The substrates were added in neutral solution and the arginase and urease activity were determined over a period of one hour. Two sets of experiments were carried out. Enzyme activity in the ovary and muscle was very low, but in the intestine both arginase and urease were present. Thus, when urea was added, ammonia production was increased by 68-100 μg. ammonia nitrogen per g. dry wt. per hour, and, when arginine was added, the urea plus ammonia nitrogen was increased by 160-250 μg. per g. dry wt. per hour.

TABLE 4

FORMATION OF UREA AND AMMONIA FROM THE INTERMEDIATES OF THE ORNITHINE CYCLE

Substrate Added	Ammonia plus Urea N (mg./g. dry wt.)	
	Ascaris Intestine	*Ascaridia galli*
None	0.26	0.36
Oxaloacetate	0.25	0.36
Oxaloacetate + ornithine	0.26	0.44
Oxaloacetate + citrulline	0.74	0.50
Oxaloacetate + arginine	0.78	0.68

(*h*) *The Catabolism of Purines and Related Compounds*

The effects of muscle adenylic acid, adenine, guanine, xanthine, uric acid, and allantoin on ammonia and urea production were examined. Of these compounds, only adenylic acid appreciably increased ammonia production in brei prepared from the muscle of *Ascaris lumbricoides.* Brei prepared from large *Ascaridia galli,* taken from birds that had been infected eight weeks previously, produced large amounts of ammonia from adenylic acid and smaller amounts from adenine. The other compounds were not attacked. Small *Ascaridia galli,* from five-week-old infestations, gave different results. With the exception of guanine all the compounds caused an increase in ammonia production;

uric acid and allantoin caused an increase in urea formation (see Table 5). The magnitude of activity, particularly in respect to urea formation, varied considerably in different lots of *Ascaridia galli*, but in all experiments activity was greatest when young parasites were used, and the relative activity of the different substrates was consistent.

TABLE 5

AMMONIA AND UREA PRODUCTION (MG./G. DRY WT.) FROM PURINES AND THEIR DERIVATIVES

Substrate Added	Large *A. galli*		Small *A. galli*	
	Ammonia N	Urea N	Ammonia N	Urea N
None	0.16	Trace	0.19	0.04
Guanine	0.16	Trace	0.19	0.03
Adenine	0.22	Trace	0.21	0.04
Adenylic acid	0.40	Trace	1.58	0.05
Xanthine	0.16	Trace	0.36	0.06
Uric acid	0.16	Trace	0.37	0.68
Allantoin	0.16	Trace	0.51	0.91

IV. DISCUSSION

The partition of nitrogen in the excreta of *Nematodirus* spp. and *Ascaridia galli* is shown in Table 1. The predominance of ammonia over urea and uric acid as the chief excretory product is similar to that found in annelids (Delaunay 1931; Bahl 1947; Cohen and Lewis 1949), echinoderms (Delaunay 1931), Crustacea (Delaunay 1931; Dresel and Moyle 1950), teliosts, and Dipnoi (Scheer 1948), protozoa (Nardone and Wilber 1950), and aquatic reptiles (Coulson, Hernandez, and Brazda 1950; Khalil 1947). Urea forms the major excretory product of mammals and Amphibia; uric acid predominates in the excreta of birds, terrestrial reptiles, and insects.

The nature of the end products of nitrogen metabolism is of considerable biological significance (Delaunay 1931; Needham 1931, 1942). Though a large part of the excreted nitrogen in all animals has a common origin, namely the α-amino acid nitrogen of the food proteins, the excretory catabolism may take any of several different forms. Thus, as indicated above, uricotelism is a characteristic of terrestrial animals that live under conditions of water shortage and have cleidoic eggs; ureotelism is found among animals that do not suffer acute water shortage and are viviparous or produce non-cleidoic eggs laid in humid environments; ammonotelic organisms live in environments providing sufficient water to allow the ammonia concentration in their tissues to be kept below a toxic level.

The ammonotelic character of the nitrogen catabolism of nematode parasites of the alimentary canal is in accordance with the fact that water is freely available from the environment. Though the osmotic pressure of the contents of the digestive tracts of most animals is high compared to serum and tissue, the osmotic pressure of the fluids and tissues of the parasites is even higher

(Schopfer 1932) and there is, therefore, an osmotic gradient tending to drive water into the parasites. Ammonia is, however, highly toxic to nematode parasites (von Brand and Simpson 1947) and it is probable that a lowering of the rate of water exchange between the parasites and the environment would soon lead to the accumulation of toxic amounts of ammonia in the tissues. Some free-living stages of nematodes, e.g. third-stage larvae of *Trichostrongylus*, probably live under conditions of water shortage and it would be expected that uric acid would form the major nitrogenous excretory product.

The production of urea, which formed an appreciable proportion of the total nitrogen excreted (see Table 1), was much decreased when *Nematodirus* spp. were kept under anaerobic conditions. It is known that the synthesis of urea in mammalian tissues is an endergonic process and that the amounts produced are increased in the presence of lactate, or adenosinetriphosphate, and oxygen (Krebs and Henseleit 1932; Cohen and Hayano 1948). If an appreciable proportion of the energy requirements of *Nematodirus* spp. is satisfied by aerobic mechanisms (Rogers 1949) it would be expected that the production of urea might decrease under anaerobic conditions. On the other hand *Ascaridia galli*, in which urea production was not reduced in the absence of oxygen, is largely anaerobic *in vivo* (Rogers 1949) and is probably better equipped to obtain energy from anaerobic mechanisms.

The material precipitated by phosphotungstic acid, but not by trichloracetic acid, forms one of the most interesting components found in the excreta of the parasites. This material has not been examined as yet, but in view of the possibility that toxins and antigens secreted by the parasites would be included in this fraction, its composition will be described in detail in a later publication. It might appear that this material was obtained from the host ingesta and represents the undigested residue from the alimentary tracts of the parasites. However, when the parasites were kept in artificial media for periods greater than 24 hours this fraction tended to increase rather than to decrease. It is probable then, that it was a true excretory product, perhaps arising from the cells of the intestines of the parasites, or from the reproductive organs.

The total amount of the soluble nitrogen compounds excreted by *Nematodirus* spp. was large compared with that of many invertebrates. For instance, the nitrogen excreted by fasting Crustacea examined by Dresel and Moyle (1950) ranged from the low level of the terrestrial *Oniscus asellus*, 0.03 mg. per g. wet wt. per 24 hours, to that of the estuarine form *Gammarus zaddachi*, 0.60 mg. per g. per 24 hours. The level of nitrogen excretion in *Ascaridia galli* was a third to a sixth of that of *Nematodirus* spp. and was nearer to that of *Ascaris lumbricoides*, 0.15-0.29 mg. per g. wet wt. per 24 hours (Weinland 1904; von Brand 1934). The high level of nitrogen excretion in *Nematodirus* spp., compared with *Ascaridia galli*, suggests that it may have utilized protein as a source of energy to a greater extent. The respiratory quotient of *Ascaridia galli* indicates that carbohydrate is the chief source of its energy; the low respiratory quotient of *Nematodirus* spp. (Rogers 1948) indicates that important substrates besides carbohydrate may be oxidized to provide energy.

The ammonia found in the media in which parasites had been kept may have been due to the action of urease or deaminases. The results obtained indicate that adenylic deaminase and adenase were present in the parasites' tissues. No guanase activity was found. It is possible that some ammonia was produced directly by the action of amino acid deaminases. Urease was present in all the parasites examined. *Haemonchus contortus* was examined especially for urease activity because Glick, Zak, and von Korff (1950) suggest that the production of ammonia by urease in the mucosa of the stomach may assist in protecting the tissue from acid and from pepsin. This parasite lives in the abomasum of the sheep and would need some mechanism to protect it from the host's gastric juices. However, the results obtained indicate that urease was not present in sufficient amounts to warrant the suggestion that it has a protective function in *Haemonchus contortus.*

The substrate for urease may have been provided from ingested urea, urea formed by the ornithine cycle, or from the catabolism of purines. It is possible that the urea found in the culture media could have come from any of these sources. The catalytic action of ornithine, citrulline, and arginine on urea production suggests that ammonia arising from amino acid deamination was probably captured in the cyclical process, ornithine + carbamyl glutamate → citrulline → arginine → ornithine + urea (Ratner 1949). This ornithine cycle, or something similar to it, has been found to function in the liver of the rat and other mammals, and in the tortoise and the frog. Recent work (Cohen and Lewis 1950) has shown that the ornithine cycle also functions in the tissues of the intestine of *Lumbricus terrestris.* This mechanism of urea synthesis is not found in the livers of birds or snakes.

The results shown in Table 5 show that urea was formed in the parasites by the breakdown of purines. It is probable that the route taken was similar to that suggested by Florkin and Duchateau (1943), and involved the successive action of urico-oxidase, allantoinase, and allantoicase. The conversion of small amounts of xanthine to uric acid by the action of xanthine oxidase would account for the urea formed from this substrate.

The complete catabolism of purines to urea, and finally to ammonia, as probably occurs in the tissues of some nematode parasites, has been found in marine lamellibranchs, sipunculids, and Crustacea (Florkin 1949). In other animals the breakdown may stop at urea, allantoic acid, or allantoin; or uric acid may not be attacked at all.

The difference in the rate of purine catabolism of tissue preparations from young and old *Ascaridia galli* was pronounced and it seems that certain metabolic activities of the parasites are suppressed or lost as they grow larger. It is possible that this represents a simplification of metabolism, which took place as the parasitic mode of life became more firmly established.

V. Acknowledgments

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THE CELL WALL STRUCTURE OF XYLEM PARENCHYMA

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Summary

The fine structure of the cell wall of both ray and vertical parenchyma has been investigated. In all species examined secondary thickening had occurred. In the primary cell wall the micellar orientation was approximately transverse to the longitudinal cell axis. Using optical and X-ray methods the secondary cell wall was shown to possess a helical micellar organization, the micelles being inclined between 30° and 60° to the longitudinal cell axis.

The distribution of the various cell wall constituents was studied, and it was observed that most of the lignin occurred in the intercellular layer and primary wall. The layered structure of the cell wall of sclerosed parenchyma cells was also investigated and it was demonstrated that these cell walls possess both structural and chemical heterogeneity.

I. Introduction

In previous investigations of the cell wall structure of tissue elements of the xylem, attention has centred mainly on elements of mechanical or conducting function. The fine structure of xylem parenchyma—ray and vertical parenchyma—has received little attention. In form, the individual parenchyma cells are isodiametric or elongated with truncated ends and are usually thin-walled in comparison with the prosenchyma elements. They possess simple pitting. The fine structure of ray parenchyma appears to have been first studied by Tuszon (1903) who observed helical fractures in the walls of isolated cells. These he considered to be evidence of a helical micellar organization in the cell wall. A similar conclusion was reached much later, from optical (Ritter and Mitchell 1939) and X-ray (Gross, Clarke, and Ritter 1939) studies of the ray parenchyma of gymnosperms. So far as the writers are aware, no study has been made of the distribution of the cell wall constituents in ray or vertical parenchyma nor has any direct evidence of their cell wall organization been presented, apart from the work of Tuszon. Accordingly, the investigations described below were undertaken.

II. Materials and Methods

Various species with comparatively thick-walled ray and vertical parenchyma cells were selected for investigation, particular attention being paid to the 'sclerosed' cells in vertical parenchyma and 'stone' cells in rays where these occurred. Such cells have unusually thickened cell walls and are similar in

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some features to the stone cells of phloem. Sclerosed tyloses were also examined since they are developed from parenchyma cells (Chattaway 1949). The species forming the main basis of the investigations reported here were:

Dialium laurium Baker (Leguminosae)—sclerosed and thin-walled ray and vertical parenchyma.

Fagus atropunica Sudw. (Fagaceae)—ray parenchyma.

Grevillea robusta A.Cunn. (Proteaceae)—ray parenchyma.

Gymnacranthera farquhariana Warb. (Myristicaceae)—sclerosed tyloses.

Hodgkinsonia ovatiflora F.Muell. (Rubiaceae) — ray and vertical parenchyma.

Persoonia lanceolata Andr. (Proteaceae)—'stone' cells in rays.

Podocarpus amara F.M.Bailey (Podocarpaceae)—ray parenchyma.

Pongamia pinnata L.Merr. (Leguminosae)—vertical parenchyma.

Tsuga canadensis L.Carr (Pinaceae)—ray parenchyma.

Using thin sections of this material the distribution of lignin in the cell wall was examined by the staining technique of Coppick and Fowler (1939) and the 72 per cent. sulphuric acid method (Dadswell 1931). The presence of polyuronide substances in the cell wall was demonstrated by treatment of carefully delignified sections with cuprammonium solution (Kerr and Bailey 1934), the delignification being effected by alternate treatments with chlorine water and 3 per cent. alcoholic monoethanolamine (van Beckum and Ritter 1937).

In cell walls with helical micellar organization it is customary to describe the micellar orientation by reference to the angle made by a tangent to the helix and the major morphological axis of the cell. This angle is referred to in the remainder of this paper as the 'micellar angle,' and can be determined by a number of methods to which further reference will be made. From species with wide rays, e.g. *Grevillea robusta*, or with wide bands of parenchyma, e.g. *Pongamia pinnata*, it was possible to dissect out bands of ray or vertical parenchyma tissue free from other elements. These were used for direct X-ray examination. For species with narrow rays or narrow bands of parenchyma the technique of building up a composite specimen from thin sections was employed (see Gross, Clarke, and Ritter 1939) before X-ray examination.

The X-ray diagrams obtained in the present study varied considerably. From *Grevillea robusta* ray cells, good although faint 4-point diagrams were obtained, permitting reasonably accurate determination of the micellar angle. Usually, however, 2-point diagrams consisting of two diffuse arcs were obtained as in *Pongamia pinnata*. Estimates of the micellar angle from these diagrams would be over-estimates compared with values obtained by other methods. The diffuseness of these diagrams may arise because of the extremely thin specimens used, and the existence of angular dispersion of the micelles about the direction of preferred orientation.

By optical methods the micellar angle can be determined from the position of the major extinction position (see, for example, Hartshorne and Stuart 1950) or from the value of the birefringence of the cell wall in transverse section. The second method was of particular value when the cell wall was very thick and the micellar orientation varied in the different layers, when the pitting of the cell wall made the determination of the major extinction position difficult, or when the X-ray diffraction pattern was too diffuse to permit measurement. It must be emphasized, however, that the results obtained by the birefringence method were only approximate as several assumptions were necessary in the calculations.

The birefringence, $(n'_\gamma - n_\alpha)$, of the cell wall in transverse section was determined using the de Senarmont compensator (Ambronn and Frey 1926; see also Hartshorne and Stuart 1950) and was calculated from the relation

$$n'_\gamma - n_\alpha = \phi\lambda/360d, \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where

ϕ is the phase difference (°),

λ is the wavelength of the light (μ) (0.59 for the Na line),

and d is the section thickness (μ).

After measuring the phase difference the section thickness was determined. This was done by macerating the section on a slide and turning the cells on their sides for direct measurement by gently sliding the cover glass (Preston 1946).

As the cell wall is not entirely composed of cellulose, the birefringence of which must be known in order to calculate the micellar orientation, it is necessary to make an estimate of the birefringence of a body the composition of which is approximately that of the parenchyma cell wall. Harlow and Wise (1928) have published figures for the cellulose content of ray parenchyma varying from 32 to 42 per cent. In the calculations made in this investigation it was assumed that the cellulose content was 40 per cent. If this assumed value is too high in comparison with the actual value for the specimen examined, then the calculated micellar angle will be too small, and conversely if the assumed value is too low the calculated angle will be too high.

The birefringence of a body containing 40 per cent. cellulose can be calculated from the mixture formula of Hermans (1949):

$$v_m\,(n_m - 1) = v_1(n_1 - 1) + v_2\,(n_2 - 1) + \quad \ldots \quad (2)$$

where v_1, v_2 . . . . . are the volumes; n_1, n_2 . . . . . the refractive indices of the components; and v_m and n_m the corresponding quantities for the mixture. Thus, if $n_{\gamma 1}$ and $n_{\alpha 1}$ are the major and minor refractive indices of cellulose, n_γ and n_α the corresponding quantities for a body containing 40 per cent. cellulose, and n_R the refractive index of the non-crystalline components of the cell wall, then, by applying equation (2) separately to each of the refractive indices of cellulose, the following equations are obtained:

$$v_m\,(n_\gamma - 1) = v_1\,(n_{\gamma 1} - 1) + v_2\,(n_R - 1) \quad \ldots \quad (3)$$

$$v_m\,(n_\alpha - 1) = v_1\,(n_{\alpha 1} - 1) + v_2\,(n_R - 1). \quad \ldots \quad (4)$$

Subtracting equation (4) from equation (3) and rearranging:

$$n_\gamma - n_\alpha = \frac{v_1\,(n_{\gamma 1} - n_{\alpha 1})}{v_m} \quad .. \quad .. \quad (5)$$

Taking the density of the cellulose as 1.56 and that of the cell wall as 1.46 (Stamm and Hansen 1937), $v_1 = 0.4/1.56$, $v_m = 1/1.46$, and $n_{\gamma 1} - n_{\alpha 1} = 0.07$ (Hermans 1949), then $n_\gamma - n_\alpha = \frac{0.4 \times 1.46}{1.56} \times 0.07 = 0.026$. For a cell wall containing 40 per cent. cellulose and a birefringence of $n'_\gamma - n_\alpha$ in transverse section, it can be seen from the section of the index ellipsoid shown in Figure 1 that OA $= n_\gamma$, OB $= n_\alpha$, OC $= n'_\gamma$ (Preston 1946; Preston and Wardrop 1949).

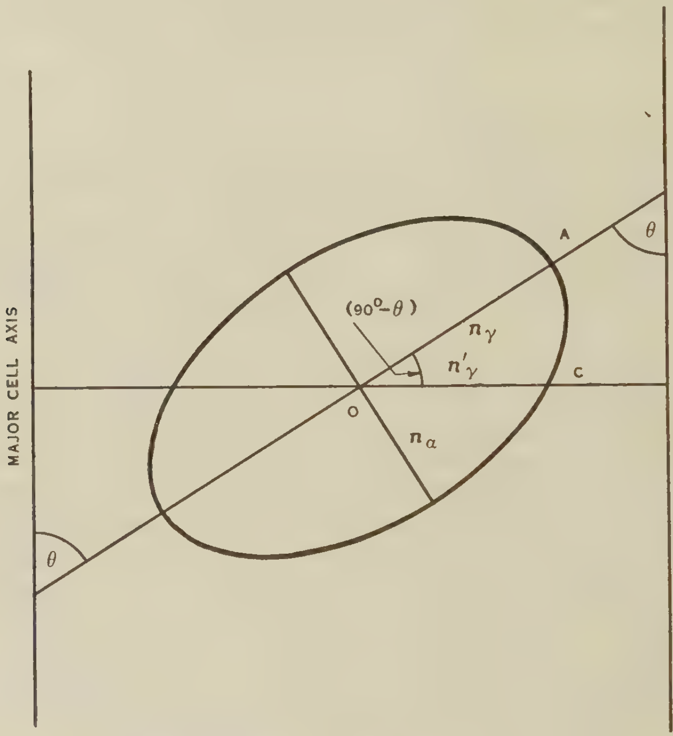

Fig. 1.—For explanation, see text.

From the geometry of the ellipse,

$$\frac{n'^2_\gamma \sin^2\theta}{n_\gamma^2} + \frac{n'^2_\gamma \cos^2\theta}{n_\alpha^2} = 1,$$

whence

$$\cos\theta = \frac{n_\alpha}{n'_\gamma}\left[\frac{(n_\gamma - n'_\gamma)(n_\gamma + n'_\gamma)}{(n_\gamma - n_\alpha)\,(n_\gamma + n_\alpha)}\right]^{\frac{1}{2}}. \quad .. \quad (6)$$

As pointed out by Meredith (1946), n_α is regarded as being approximately unchanged at different values of θ as the micelles are oriented in layers parallel

to the cell surface. Values of θ for various specimens obtained by using equation (6) are given in Table 1.

TABLE 1

BIREFRINGENCE IN TRANSVERSE SECTION AND MICELLAR ANGLES OF XYLEM PARENCHYMA

Species	Birefringence in Transverse Section $(n'_\gamma - n_\alpha)$	Micellar Angle (degrees)
Dialium laurium		
Ray parenchyma	0.013	45
Grevillea robusta		
Ray parenchyma	0.007	32
Hodgkinsonia ovatiflora		
Ray parenchyma	0.007	32
Vertical parenchyma	0.007	32
Pongamia pinnata		
Vertical parenchyma	0.015	50

III. Results and Discussion

(a) Distribution of Cell Wall Constituents

In all specimens examined the cell wall of both vertical and ray parenchyma had undergone secondary thickening. This was very slight in *Podocarpus amara*, especially in the vertical parenchyma, and was poorly developed in *Pongamia pinnata*, but in the other species the secondary wall was readily recognizable. The primary wall was discernible only with great difficulty in cross sections of cells *in situ*, but could be detected when carefully delignified (holocellulose method) sections were examined between crossed nicols. Plate 1, Figure 1, shows the primary wall of the ray cells of *Grevillea robusta* after this treatment.

Using the staining technique of Coppick and Fowler (1939) on sections cut transverse to the long axis of ray and vertical parenchyma cells, the lignin was observed to be situated mainly in the intercellular zone and the primary wall, although the secondary wall was also strongly stained. However, when the sections were treated with 72 per cent. sulphuric acid no coherent lignin residue from the secondary wall was obtained, but the primary wall and intercellular zone were so heavily lignified that they left a lignin framework undistorted by the considerable swelling of the secondary wall by the acid. The lignin framework so obtained from ray parenchyma of *Grevillea robusta* is shown in Plate 1, Figure 2, and from the vertical parenchyma of *Pongamia pinnata* in Plate 1, Figure 3.

The presence of polyuronide or pectic substances in the primary wall was demonstrated by treating carefully delignified sections with cuprammonium solution following the method adopted by Kerr and Bailey (1934). Plate 1, Figure 4, shows the result of this treatment on the ray parenchyma of *Grevillea*

robusta. It is of interest to note that the polyuronide residual layer is much thicker than that obtained by similar treatment of cross sections of fibres. The pectic or polyuronide nature of the framework was established from the fact that no residue was observed when the delignified sections were extracted with ammonium oxalate, or hydrolysed by dilute acid, before treatment with the cuprammonium solution. These observations on xylem parenchyma, together with those of Kerr and Bailey (1934) on fibres and tracheids, demonstrate the essential similarity in chemical composition of the different cells of the xylem.

(*b*) *Cell Wall Organization*

The micellar orientation in the primary wall of the ray parenchyma of *Grevillea robusta* was determined from the direction of the major extinction position and was found to be inclined at an angle of 80°-90° to the long axis of the cell. Material for examination in this way was obtained by gently tearing delignified radial longitudinal sections along the direction of the rays when fragments of the primary wall remained adhering to the untorn cells. Using this technique the micellar orientation of the primary wall of vertical parenchyma cells in *Pongamia pinnata* was also found to be 80°-90° to the long axis of the cell. Thus the micellar orientation of the primary wall of parenchyma is similar to that in the primary wall of tracheids (Preston 1947).

In sections cut at right angles to the length of the parenchyma cells the secondary wall of both vertical and ray parenchyma when viewed between crossed nicols appeared optically homogeneous in the species of *Pongamia, Grevillea, Hodgkinsonia,* and *Podocarpus* examined; this was also the case in the vertical parenchyma of *Fagus* and the ray parenchyma of *Tsuga.* Examples of this are shown in Plate 1, Figures 5 and 6, for the ray parenchyma of *Grevillea robusta* and vertical parenchyma of *Pongamia pinnata* respectively. However, in the ray parenchyma of *Dialium laurium* (Plate 2, Fig. 2) and of *Fagus atropunica* (Plate 2, Fig. 1) there was evidence of a layered structure in the secondary wall. This was also apparent in the vertical parenchyma of *Dialium laurium* (Plate 2, Fig. 3) and was greatly pronounced in 'sclerosed' vertical parenchyma cells of this species (see Plate 4, Fig. 1).

As indicated earlier, Tuszon (1903) referred to the presence of a helical organization of the cell wall of rays. Direct demonstration of this type of organization was attempted using the technique of crushing isolated cells previously described by the authors (Wardop and Dadswell 1948). The application of this technique was more difficult with parenchyma cells than with fibres because of the presence of numerous pits in the relatively thin cell wall. However, in *Grevillea robusta* it was found to be possible and from Figure 4 of Plate 2 the helical organization of a ray parenchyma cell is quite apparent. It was assumed that the cell wall tended to cleave parallel to the direction of micellar orientation, because of the 'fibre-like' texture of the secondary wall in which there exists little dispersion of the micelles about their direction of orientation. The angle of the striations so produced was found to be approximately 38°. In view of the distortion which inevitably occurs when using

this technique the result is in quite good agreement with the angle of 40° obtained for the micellar orientation by X-ray methods, and the angle of 33° from the birefringence measured on cross sections of ray parenchyma cells from the same source.

Further information on the helical organization of ray parenchyma cells from *Grevillea robusta* was obtained from a study of the end walls of the cells. This was accomplished by cutting a series of sections 8 μ in thickness, at right angles to the long axis of the ray cells, from which two sections containing the end walls of contiguous cells were selected. In the first section the end wall of the cell under investigation, and that of the next cell nearer the bark, were obtained, whereas in the second section the other end wall of the cell studied and that of the next cell near the stem centre were obtained, so that both end walls of the cell investigated were available. These sections were carefully delignified, taking care that their orientation relative to each other in the ray was not altered. Upon subsequent treatment with 0.1 per cent. sodium hydroxide the end walls of the contiguous cells were separated by gently sliding the cover glass and the two end walls of the one cell were available for examination.

The optical behaviour of the end walls was complicated by the presence of numerous pits. When viewed between crossed nicols the micelles in some walls were apparently oriented uniformly in one direction. In most walls studied, however, there was evidence that the micellar orientation was not uniform. Thus, on rotation of the specimen through 360° there were four positions of maximum brightness (Plate 2, Fig. 5) indicating the presence of strands of cellulose of uniform orientation. Upon reorienting the specimen so that these strands were in a position of extinction, extinction of the whole field did not occur, but the area between the strands appeared bright (Plate 2, Fig. 6). This indicated the presence of more than one direction of micellar orientation in the end walls. This could be demonstrated further when the specimen was examined between crossed nicols with the addition of a retardation plate (first order). When the specimen was placed so that the strands of cellulose, shown in Plate 2, Figure 5, were parallel to the direction of the slow ray of the plate, the strands gave a blue addition colour, whereas the regions between them gave an orange-red subtraction colour. These colours were reversed on rotating the stage 90°.

In these end walls it is necessary to consider whether the heterogeneity arises as a result of a gross distortion by the pits of an otherwise uniform orientation or whether in fact there exist several directions of micellar orientation in the wall, i.e. a crossed fibrillar type of structure. Evidence of the latter possibility is contained in the observation that when viewed between crossed nicols the points where the different micellar directions apparently cross do not undergo complete extinction, and also the direction of elongation of the pits in the end walls is not uniform (Plate 2, Fig. 7). Confirmation of this evidence was sought by crushing isolated end walls after staining with

congo red in the manner described for the lateral walls. This was unsuccessful as the walls tended to fragment on crushing and no conclusive observations were made. However, some evidence of a crossed fibrillar structure was observed in some cases near the edges of the end wall where there were two sets of striations intersecting at an acute angle (Plate 2, Fig. 8).

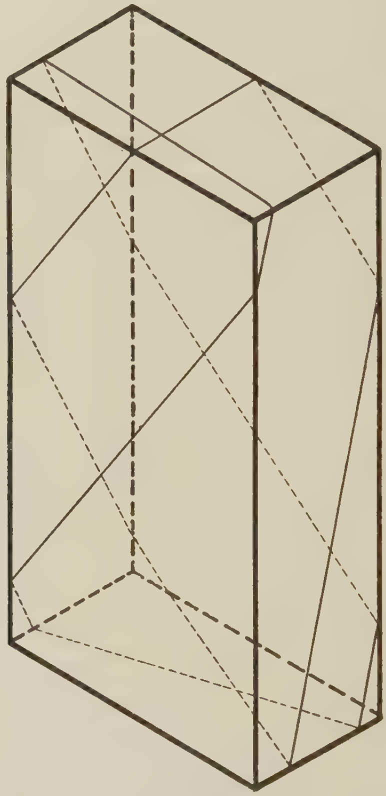

Fig. 2.—A diagrammatic representation of the supposed micellar orientation in ray parenchyma. Two loops are considered to have been wound on a rectangular prism to give a uniform helical arrangement on the lateral walls and to cross on the end walls.

Admittedly the evidence of a crossed micellar orientation of the end wall is inconclusive. However, such a structure must be considered in relation to the way in which it can be reconciled with the uniform helical micellar organization of the lateral walls. In this respect the model proposed by Preston (1934) made by winding a series of loops on a rectangular prism is of assistance. The loops may be wound so that they are parallel to each other on the end walls (as considered by Preston) and this would correspond to the examples observed with apparently uniform orientation on the end walls; or they may be wound with the loops crossing on the end walls as shown diagram-

matically in Figure 2. This would correspond to the examples with an apparently crossed fibrillar type of structure. Such models are essentially similar and possess no centres of organization or 'poles,' as observed by Preston and Astbury (1937) in *Valonia,* and thus seem to represent an essentially different type of organization.

The helical organization of the secondary wall in ray parenchyma cells was further demonstrated in cells isolated from a specimen of *Hodgkinsonia ovatiflora* which had been attacked by fungus. Cells of ray and vertical parenchyma and a vessel element from the specimen are shown on Plate 3, Figures 1-3. In these phase-contrast photomicrographs the light areas are those from which cellulose had been removed. The direction of the cavities in the cell wall was parallel to that of the major extinction position in each case. For the ray cell in Plate 3, Figure 1, the inclination of the cavity to the longer cell axis was approximately 40°, which is in fair agreement with the angle of 33° calculated from the birefringence of the intact cell wall in cross section. Bailey and Vestal (1937) have described fungal penetration of the cell wall parallel to the direction of micellar orientation, the cavities so produced having pointed ends (cf. Plate 3, Fig. 2).

Using the birefringence method the micellar orientation in normal ray parenchyma cells of *Dialium laurium* has been calculated to be 50°, and of *Podocarpus amara* to be 40°. Thus in the ray cells investigated the micellar angle in the secondary wall varied between 30° and 50°.

The organization of the primary wall in vertical parenchyma was demonstrated in the manner described earlier for ray parenchyma and similar results were obtained. The organization of the secondary wall was again studied by optical and X-ray methods but, because of its thinness, attempts to demonstrate the helical organization by the technique of crushing isolated cells were unsuccessful. However, the presence of a helical organization was clearly demonstrated in cells of *Hodgkinsonia ovatiflora* which had been attacked by fungus. The cavities in the cell walls were inclined at an angle of 30° to the longer cell axis, and these cavities were parallel to the major extinction position. From the birefringence of unattacked cells in transverse section the angle was calculated to be 33°.

The micellar angle in parenchyma cells of *Pongamia pinnata* was found to be 60° to the longitudinal cell axis from the measurement of the major extinction position, and 54° by birefringence measurements on cross sections. X-ray methods, using carefully isolated strands containing parenchyma cells only, gave a value of 55°. The micellar angle of normal parenchyma cells in *Dialium laurium* was 55° calculated from birefringence measurements.

In the various examples studied the cell wall organization of both ray and vertical parenchyma cells is essentially the same in distribution of chemical constituents, helical micellar arrangement in the secondary wall, and magnitude of the micellar angle. The relatively large micellar angles observed are of interest, particularly in the ray parenchyma, in relation to the influence attributed to these cells in governing the shrinkage in wood (Greenhill 1936). The

difficulty of determining the exact micellar angle in individual cells militated against the establishment of any relationship between length and micellar angle such as has been established for conifer tracheids by Preston (1934, 1948).

(c) *Sclerosed Parenchyma*

In addition to the cells with comparatively thin walls the investigation of which has been referred to above, there occur in both rays and vertical parenchyma of certain species some cells with greatly thickened cell walls. These cells are similar in some features to the 'stone' cells of phloem and other tissues; they have in fact been referred to under this name when observed in xylem ray tissue. The more general term, however, is 'sclerosed parenchyma.' Sclerosed parenchyma cells selected for investigation were from the vertical parenchyma strands of *Dialium laurium* and the ray tissues of *Persoonia lanceolata.* The presence of the primary wall was demonstrated in the manner described above for normal ray cells of *Grevillea robusta.*

In the vertical parenchyma of *Dialium laurium* it was observed that the secondary wall consisted of a number of layers distinctly visible when thin cross sections were viewed in the polarizing microscope between crossed nicols (Plate 4, Fig. 1). Each layer was composite and in cross section appeared to consist of an outer, thicker, birefringent part and an inner, narrower, less birefringent or isotropic part. The layers were separated from each other by delignifying longitudinal sections cut sufficiently thin to include no whole cells (8 μ), and then gently crushing the section by sliding a cover glass over it (Plate 3, Fig. 4). When these transverse sections were treated with 72 per cent. sulphuric acid the secondary wall of the sclerosed parenchyma cells swelled greatly, revealing that each layer consisted of a number of lamellae 0.1-0.2 μ in thickness and of varying lignin content (see Plate 3, Figs. 5 and 6). In addition, between the individual layers was a thin isotropic lamella consisting mainly of lignin (Plate 3, Fig. 5). The presence of a non-cellulosic lamella separating physically discrete layers of the cell wall has also been recorded for fibres of bamboo by Lüdtke (1931, 1934) and for *Pandanus* by Bailey and Kerr (1935).

As well as variations in the chemical nature of the walls of sclerosed parenchyma cells there are also variations in their physical nature. This can be seen from the optical heterogeneity in transverse section (Plate 4, Fig. 1), and in longitudinal section (Plate 4, Fig. 2). From these photomicrographs it is apparent that the part of the wall appearing birefringent in transverse section is isotropic or feebly birefringent in longitudinal section, and the part isotropic in transverse section appears birefringent in longitudinal section. From these observations it was concluded that within each layer the optical heterogeneity observed can be attributed to a difference in micellar orientation. That each layer consists of a number of lamellae may be attributed to variations in micellar texture such that in the lignin-rich lamellae the volume of the intermicellar system is greater than in the lignin-poor lamellae.

Further information concerning the differences in micellar orientation between layers as distinct from the differences within them described above can be obtained from the study of birefringence or phase difference in successively formed layers. Changes observed when examining cross and longitudinal sections of typical cells with thickened secondary walls are shown in Table 2. It will be noted that in the transverse section the phase difference of the birefringent layers decreased in successively formed layers (i.e. in each layer nearer the lumen) and that in the longitudinal section the phase difference also decreased towards the lumen. Assuming this change in phase difference reflects a change in orientation rather than in composition then the micellar angle would be expected to decrease in each successively formed layer appearing birefringent in transverse section.

TABLE 2

EVIDENCE OF DIFFERENCES IN MICELLAR ORIENTATION IN VARIOUS LAYERS OF THE CELL WALL OF SCLEROSED VERTICAL PARENCHYMA OF *DIALIUM LAURIUM*.

Layer Number From Lumen		Transverse Section			Longitudinal Section
		Phase Difference	Birefringence ($n'_\gamma - n_\alpha$)	Micellar Angle	Phase Difference
3	Outer	68	0.019	59	—
	Inner	—	—	—	40
2	Outer	56	0.016	52	—
	Inner	—	—	—	20
1	Outer	48	0.014	48	—
	Inner	—	—	—	14

The micellar angle of each of these successive birefringent layers was calculated from the magnitude of their birefringence (Table 2). On the other hand, the decrease in phase difference in successive birefringent layers in longitudinal section would be expected to reflect an increase in micellar angle with respect to the longitudinal cell axis. Unfortunately no calculation of these angles was possible because the section thickness could not be determined accurately. Thus the changes in micellar orientation between layers correspond to those that would occur if there were a constant angle between the two directions of orientation within each layer of the wall such that any decrease in the micellar angle of one orientation would imply an increase in the other.

It is of particular interest that the layered structure described above appears to resemble closely that of the cell wall in fibres of *Pandanus* (Bailey and Kerr 1935) and bamboo (Lüdtke 1931, 1934; Preston and Singh 1950). Preston and Singh concluded that in bamboo the micellar angle decreased in the successive layers appearing birefringent in transverse section but did not determine the orientation of the non-birefringent layers. A similar type of structure also appears to exist in the fibres of *Calamus* and *Cocos*, and von Mohl in 1844 (see Braun 1853) described the way in which the cell wall layers in these fibres

could be separated from each other by treatment with dilute sulphuric acid. He pointed out further that each layer consisted of an outer, broader, softer part; and an inner, thinner, firmer part.

The structure of the sclerosed parenchyma cells of *Dialium* differs from that of *Pandanus* in that the lignin is distributed in lamellae throughout each of the cell wall layers which in turn are separated by an isotropic layer of lignin, whereas in *Pandanus* the lignin is confined for the most part to the region between layers. Since the meristem giving rise to monocotyledonous fibres develops from cells which have already become parenchymatous, it is interesting to consider whether the layered type of structure observed in *Dialium* parenchyma is characteristic of all thick-walled cells of parenchymatous origin. Examination of the sclerosed ray cells (stone cells) of *Persoonia lanceolata* (Plate 4, Fig. 3) and of the sclerosed tyloses in *Gymnacranthera farquhariana*, which are of parenchymatous origin (Chattaway 1949), shows that in these cases a similar layered structure is to be found. It may be noted further that intermediate stages between the extreme simplicity of the cell wall organization of normal parenchyma cells in *Grevillea, Podocarpus*, and *Pongamia* on the one hand, and the extreme complexity of structure in the sclerosed cells of *Dialium* and *Persoonia* on the other hand, have been observed in *Fagus* (Plate 2, Fig. 1), *Dialium* (Plate 2, Figs. 2 and 3), and other genera.

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Explanation of Plates 1-4

Plate 1

Fig. 1.—*Grevillea robusta.* A transverse section of a ray after delignification and gentle tearing. Parts of the feebly birefringent primary wall (*P*) can be seen adhering to the more strongly birefringent secondary walls. x980.

Fig. 2.—*Grevillea robusta.* Part of a transverse section of a ray after treatment in 72 per cent. H_2SO_4 showing the rigid lignin framework of the middle lamella and primary wall. x430.

Fig. 3.—*Pongamia pinnata.* A transverse section of vertical parenchyma after similar treatment to the section shown in Figure 2. x430.

Fig. 4.—*Grevillea robusta.* Transverse section of a ray after delignification and treatment with cuprammonium hydroxide solution showing the polyuronide frame of the primary wall and middle lamella. x980.

Fig. 5.—*Grevillea robusta.* Transverse section of a ray between crossed nicols. x430.

Fig. 6.—*Pongamia pinnata.* Transverse section of vertical parenchyma between crossed nicols. x430.

Plate 2

Fig. 1.—*Fagus atropunica.* Transverse section of a ray between crossed nicols. x980.

Fig. 2.—*Dialium laurium.* Transverse section of ray parenchyma between crossed nicols. x980.

Fig. 3.—*Dialium laurium.* Transverse section of vertical parenchyma between crossed nicols. x980.

Fig. 4.—*Grevillea robusta.* An isolated cell of ray parenchyma after staining with congo red and crushing. Photographed in green light. x980.

Fig. 5.—*Grevillea robusta.* The end wall of a ray cell, photographed between crossed nicols, showing several strands of cellulose in the position of brightness. x1080.

Fig. 6.—*Grevillea robusta.* As in Figure 5 with the strands of cellulose appearing bright in Figure 5, in extinction. x1220.

Fig. 7.—*Grevillea robusta.* The same end wall as shown in Figures 5 and 6, stained with congo red, showing pits. x1080.

Fig. 8.—*Grevillea robusta.* The edge of an end wall after staining with congo red and crushing. Photographed between crossed nicols. x980. Total enlargement x2670.

Plate 3

Fig. 1.—*Hodgkinsonia ovatiflora.* A cell of ray parenchyma isolated by maceration showing the helical course of fungal attack. Phase contrast. Photographed x400. Total enlargement x870.

Fig. 2.—*Hodgkinsonia ovatiflora.* A cell of vertical parenchyma viewed as in Plate 3, Figure 1. Photographed x400. Total enlargement x870.

Fig. 3.—*Hodgkinsonia ovatiflora.* Part of a vessel element viewed as in Plate 3, Figures 1 and 2. Photographed x400. Total enlargement x740.

Fig. 4.—*Dialium laurium.* A single cell wall of a cell of sclerosed vertical parenchyma after delignification and separation of the various layers. x430.

Fig. 5.—*Dialium laurium.* Part of a transverse section of sclerosed vertical parenchyma after treatment with 72 per cent. H_2SO_4 showing lamellae present in each of the cell wall layers, which are separated by an isotropic layer of lignin. x980.

Fig. 6.—*Dialium laurium.* As in Plate 3, Figure 5. x980.

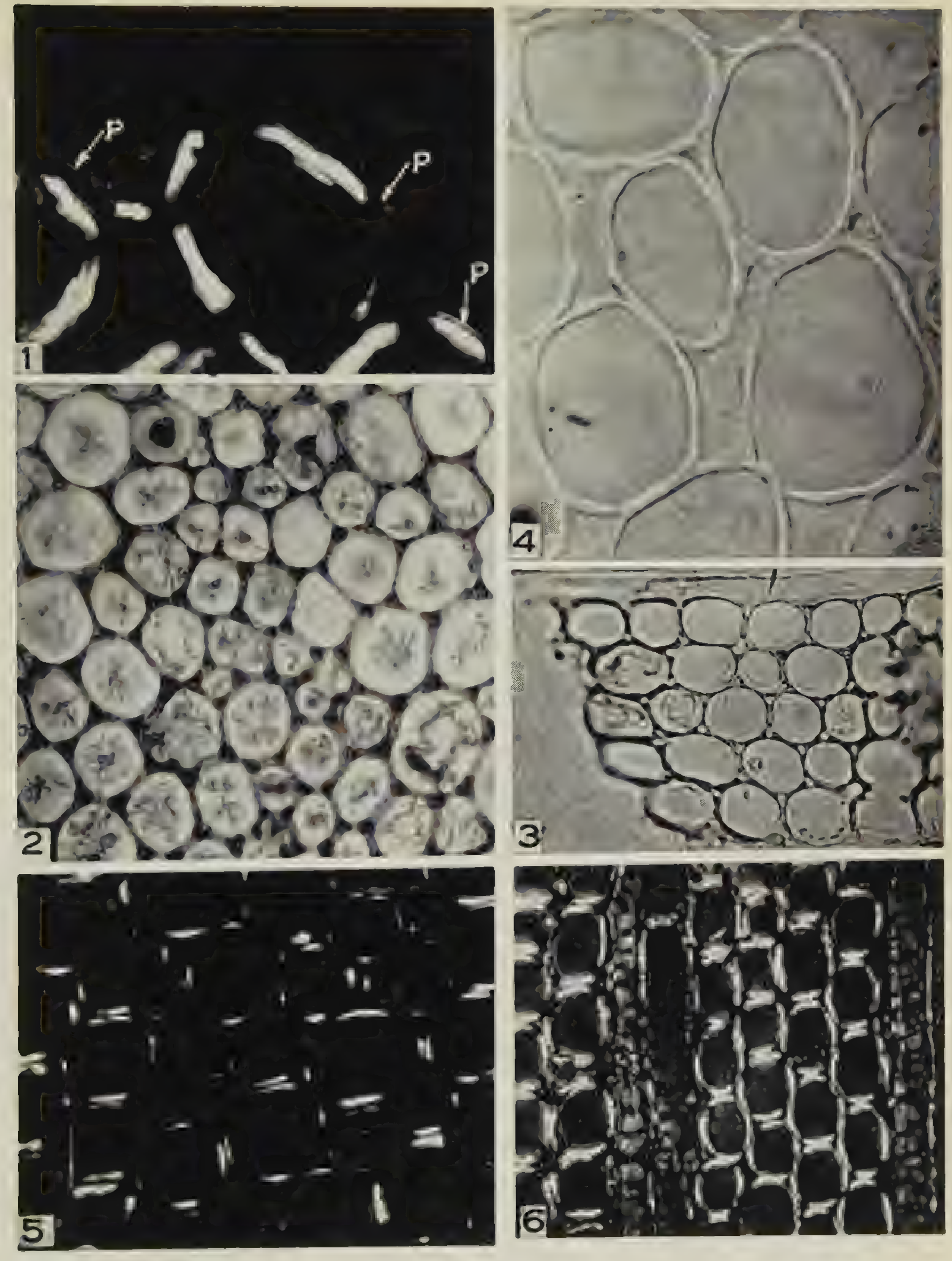
PLATE 4

Fig. 1.—*Dialium laurium.* Sclerosed vertical parenchyma in transverse section between crossed nicols. Photographed x980. Total enlargement x2250.

Fig. 2.—*Dialium laurium.* Sclerosed vertical parenchyma in longitudinal section between crossed nicols. Photographed x980. Total enlargement x2250.

Fig. 3.—*Persoonia lanceolata.* Transverse section of a sclerosed cell of ray parenchyma between crossed nicols. x980.

CELL WALL STRUCTURE OF XYLEM PARENCHYMA



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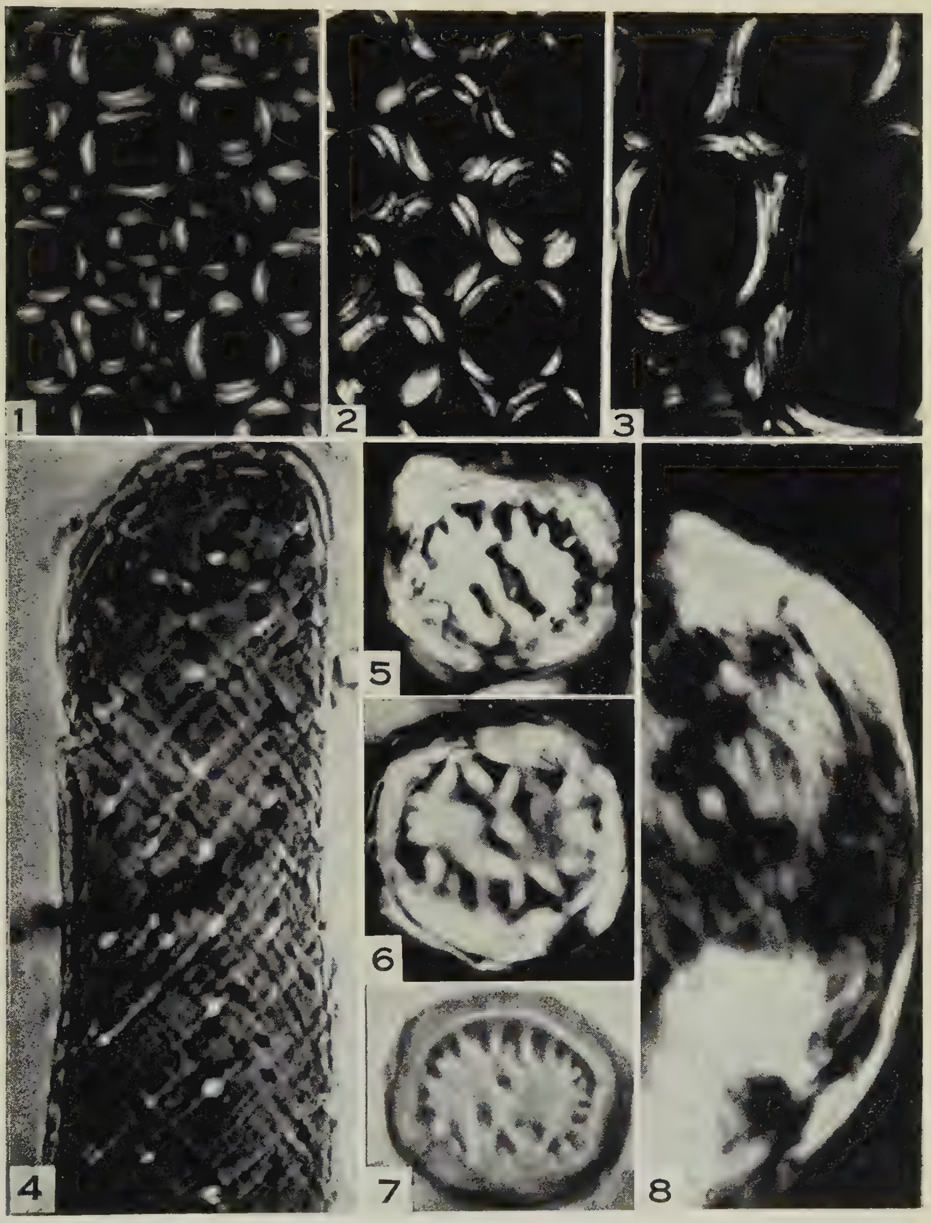

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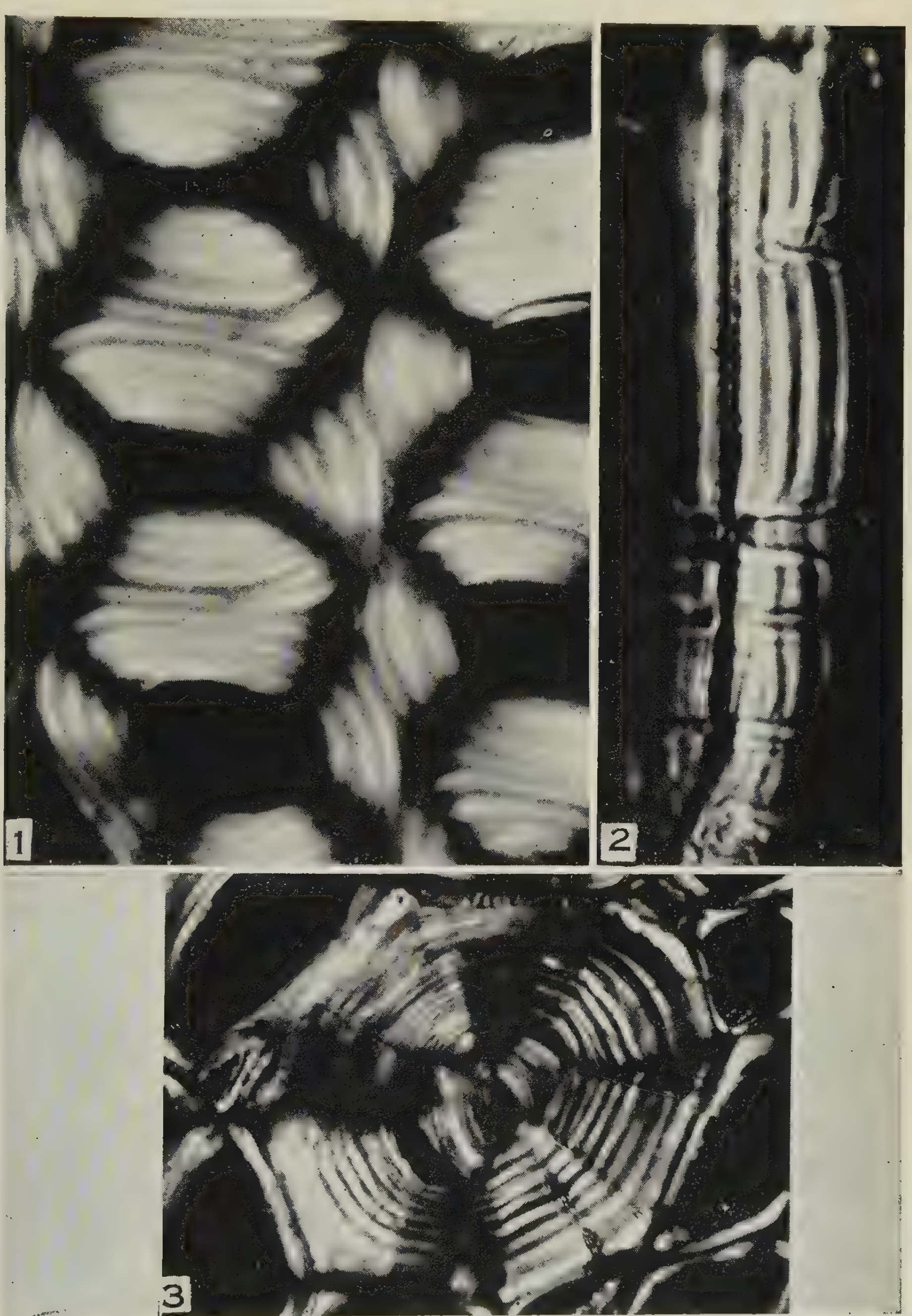

A RELATIONSHIP BETWEEN IMMUNITY AND LOCALIZED REACTION TO VIRUS X IN THE POTATO (*SOLANUM TUBEROSUM*)

By E. M. Hutton* and D. C. Wark*

[*Manuscript received September* 20, 1951]

Summary

The pattern of virus X development in the inoculated leaves of immune, localized reacting, and susceptible phenotypes in the potato has been studied. The results indicate that immunity is not absolute. Phenotypes giving a localized reaction cannot be regarded as hypersensitive. The evidence shows that an inactivating system restricts the development of the virus as soon as infection takes place.

All the results, including those from the X inoculation of first-generation seedlings, raised from intercrosses between immune, localized reacting, and susceptible phenotypes, indicate that a common virus-inactivating system determines resistance. The difference between localized resistance and immunity may be determined by different tetraploid conditions of a common major gene.

I. Introduction

Immunity to virus X in potatoes was first described by Schultz *et al.* (1934) in the American seedling U.S.D.A. 41956. Later, Stevenson, Schultz, and Clark (1939) found that in crosses between 41956 and susceptible parents, 37-42 per cent. of the progenies were immune and that selfing immune types resulted in 72-78 per cent. of the progenies being immune. On this basis they assumed two genes, one in the duplex and the other in the simplex condition, to be necessary for immunity to virus X.

Cadman (1942) studied the inheritance of the top-necrotic reaction to virus X following grafting in a number of hybrid potato progenies and found most necrotic-reacting varieties to be simplex for the dominant allele of a gene. Phenotypes reacting in this way give only localized necrotic lesions when the leaves are inoculated, and both Cockerham (1943) and Cadman (1942) regard these reactions as evidence of extreme susceptibility or hypersensitivity. Under field conditions of infection with virus X, potato varieties and seedlings with a localized reaction remain free of this virus.

Little work has been done on the nature of the immune and localized reactions to virus X in the potato, in spite of their importance in this crop and their bearing on the general problem of virus resistance. Clinch (1942) used Seedling 41956 as the stock or scion in grafts with X-infected potatoes, and also as the intermediate scion in double grafts with infected and virus-free material above and below. She found that 41956 failed to become infected

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with virus X but allowed the virus to pass unchanged through its stem. She concluded that the non-multiplication of virus X in 41956 was due to the lack in its cells of some substance or physical condition necessary for virus X synthesis. The course of infection and subsequent localization and death of the virus in hypersensitive phenotypes has not been studied. It is difficult to see how extreme susceptibility can account for the resistance of these phenotypes to systemic invasion by virus X.

At Canberra a study of the pattern of virus X development has been made in both immune and hypersensitive phenotypes. The results indicate that both reactions have a common basis and are dependent on a similar major gene.

II. Materials and Methods

A virulent strain of virus X, cultured in *Datura stramonium,* was used as described previously (Hutton 1948). The pattern of virus X development was followed in the immune 11-84, the localized reacting Epicure and 47-20, and in a virus-free clone of the susceptible Factor. The hybrids 11-84 and 47-20 derived their X resistance from 41956 and Epicure respectively.

Vigorous plants, 12 in. high were each inoculated on six leaves during the summer in the glass-house. At intervals up to 4 or 8 weeks after inoculation, according to the experiment, discs of tissue were removed from inoculated leaves with a cork borer (diam. 9 mm.). For each plant at any one interval, one disc of tissue was removed from each of the six inoculated leaves, and the discs macerated together to provide inoculum for transfers to two young *D. stramonium* plants and duplicate globe amaranth leaves (cf. Wilkinson and Blodgett 1948).

For the studies involving the inheritance of the localized and immune reactions in hybrid progenies, a series of potato crosses were made to include all combinations between selected hybrids with either immune, localized, or susceptible reactions to virus X. To prevent indirect effects on the types of inheritance, only hybrids with well-defined parental backgrounds of immunity, localized reaction, or susceptibility were selected for crossing. First-generation seedlings were raised from the hybrid seed in the autumn, and were inoculated with virus X on the third unfolded leaf from the tip when 3 in. high. Reactions were observed at weekly intervals. Seedlings that developed systemic necrosis following a localized reaction were discarded. Eight weeks after inoculation, the tips of the seedlings that had been retained were tested for the presence of virus X by transfers to globe amaranth.

III. Pattern of Virus X Development in Immune, Localized Reacting, and Susceptible Phenotypes

After the plants of 11-84, 47-20, Epicure, and Factor were inoculated, the samplings and transfers to indicators were made at intervals of 2 or 3 days for the first 2 weeks, and then at two further weekly intervals. At the final transfer, tips of all the potato plants were inoculated to the indicators.

In Table 1 the *D. stramonium* results show the presence or absence of virus X. The lesion numbers on globe amaranth indicate both presence and concentration of virus. There was a marked contrast between the different types of reaction. In the susceptible Factor, virus X quickly became established in relatively high concentration when compared with the resistant Epicure and 47-20, in which virus concentration never reached a high level, and from which virus was recovered in only a low percentage of cases. The fact that an early limited development of virus X occurred in the immune 11-84 was of considerable interest.

TABLE 1

VIRUS X RECOVERIES FROM DISCS TAKEN AT INTERVALS FROM INOCULATED LEAVES OF IMMUNE, LOCALIZED REACTING, AND SUSCEPTIBLE PHENOTYPES

A = number of potato plants out of six which gave a positive reaction on *D. stramonium**; *B* = mean number of lesions on globe amaranth from six potato plants

Intervals After Inoculation at Which Transfers Made (days)	Factor Susceptible		Epicure Localized Reacting		47-20 Localized Reacting		11-48 Immune	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
2	1	0.5	0	0.2	0	0.0	0	3.8
5	4	7.8	1	3.6	2	6.3	1	0.2
7	6	29.2	1	1.0	0	0.7	0	0.0
9	6	34.5	4	3.3	2	0.3	0	0.0
12	6	17.8	2	0.2	2	0.3	0	0.0
14	6	29.3	1	2.3	0	0.0	0	0.0
21	6	43.0	2	0.5	1	0.2	0	0.0
28	6	18.0	5	0.2	3	0.8	0	0.0
28 (Tips)	6	4.5	2	0.0	1	0.0	0	0.0

* In Factor, except for two transfers, both duplicates reacted in every case. In others, 72-100 per cent. of the transfers gave a reaction in only one of the duplicates.

The results of Table 1 give no indication of an extreme susceptibility being present in Epicure and 47-20, but rather the presence of an inactivating system which restricts virus development and concentration as soon as infection takes place. In view of the results with 11-84 it is possible that the difference between immune and localized-reacting phenotypes is only one of degree.

IV. THE IMMUNE REACTION TO VIRUS X

In view of the importance of the previous finding that the immune 11-84 allowed a very restricted development of virus X in inoculated leaves, another and similar experiment was done with this hybrid, using virus-free Factor as the control. To obviate any possibility of adhering tissue from the inoculum causing the results described, the six leaves on each of the six inoculated plants of both 11-84 and Factor were washed thoroughly with water several times after inoculation.

The results in Table 2 confirm those obtained previously. The recoveries shown for 11-84 are all from different plants, so that half the plants of this immune hybrid allowed a very restricted development of virus X, one of the recoveries being made 3 weeks after inoculation.

TABLE 2

VIRUS X RECOVERIES FROM INOCULATED LEAVES OF 11-84 AND FACTOR

		Intervals After Inoculation at Which Transfers Made (days)						Intervals After Inoculation at Which Transfers Made (weeks)							
Variety	*D. stramonium*	1	2	3	4	5	7	2	3	4	5	6	7	8	Final
11-84 (immune)	No. out of 12 which	0	0	1	0	0	0	1	1	0	0	0	0	0	0
Factor (susceptible)	reacted	0	1	2	5	7	12	12	12	12	12	12	12	12	12

V. Immunity, Resistance, and Susceptibility to Virus X Dependent on Different Tetraploid Conditions of a Common Major Gene

The experiments described have indicated that in 11-84 the immunity to virus X derived from 41956 is not absolute and is dependent on the inheritance of a highly efficient virus-inactivating system. The reaction of Epicure or 47-20, usually described as hypersensitivity, appears to be a form of general resistance dependent on the operation of a similar virus-inactivating system, in fact the difference between this reaction and immunity could well be one of degree. In order to find whether such relationship could be established genetically, segregations for the three X reactions were recorded in the hybrid progenies raised and inoculated as described in Section II. The results are given in Table 3.

The main features of Table 3 are the high proportion of localized reacting resistant seedlings from crosses involving only X-immune parents, and the appearance of immune phenotypes from crosses between localized reacting parents. These findings are further substantiated by the isolation of localized reacting phenotypes in the progeny of immune-susceptible crosses, and immune phenotypes from localized-susceptible crosses. A further point of interest is the reduction in the percentage of resistant phenotypes resulting when the immune parent was male in the two combinations, immune-localized and immune-susceptible.

These results support those obtained in the previous experiments, and establish that immune parents carry genetic factors for localized necrosis, while localized reacting ones carry factors for immunity. They also suggest that the same virus-inactivating system is being inherited by the resistant phenotypes in the various progenies. Whether this system functions as the highly

TABLE 3

PERCENTAGES OF PHENOTYPES WITH IMMUNE, RESISTANT, AND SUSCEPTIBLE REACTIONS TO VIRUS X RESULTING FROM CROSSES BETWEEN HYBRIDS WITH THESE DIFFERENT REACTIONS

Type of Parentage (Female first, Male second)	No. of Different Hybrid Progenies	No. of Seedlings Tested	Susceptible Seedlings			Resistant or Immune Seedlings			Range in Resistant or Immune Between Different Hybrid Progenies (%)
			Lesions Absent (%)	Lesions Present (%)	Total (%)	Lesions Absent (%)	Lesions Present (%)	Total (%)	
Immune × immune	5	542	25.0	26.6	51.6	12.4	36.0	48.4	29 to 56
Immune × localized	3	381	57.2	7.6	64.8	28.0	7.2	35.2	13 to 41
Localized × immune	4	425	68.5	13.6	82.1	10.1	7.8	17.9	10 to 26
Immune × susceptible	4	165	46.7	23.0	69.7	14.0	16.3	30.3	13 to 62
Susceptible × immune	4	597	75.8	19.0	94.8	4.5	0.7	5.2	3 to 25
Localized × localized	2	67	79.1	3.0	82.1	16.4	1.5	17.9	7 to 26
Localized × susceptible	3	190	64.2	14.8	79.0	14.2	6.8	21.0	14 to 31
Susceptible × susceptible	3	333	87.0	13.0	100	0.0	0.0	0.0	0

efficient immune one, or as the less effective localized necrotic one is considered to be dependent on different autotetraploid conditions of a common major gene. That using first-generation seedlings in the manner described was necessary for revealing these relationships was shown by the fact that the majority of the tuber progeny from the resistant ones gave an apparently immune reaction to virus X during the summer. It is apparent that the drastic methods used have been highly selective for immune and nearly immune phenotypes.

VI. Discussion

Some of the implications of these results have been discussed generally elsewhere (Hutton 1951). The results emphasize the need for a biochemical understanding of the virus-inactivating system involved, as this would be the only way to prove or disprove the relationships shown. There is little doubt that the term hypersensitivity is inappropriate for the reaction to X of Epicure and its derivatives, and that the term resistance should take its place. It is contended that the necrotic reaction is not necessary to the primary virus inactivation process, but is a side reaction. This is supported by the fact that the majority of resistant first-generation seedlings, which reacted necrotically, did not do so later as tuber progeny.

Cadman (1942) has summarized the evidence for the tetraploid nature of the potato. Taking into consideration his results and those of Stevenson, Schultz, and Clark (1939), as well as the ratios of resistant or immune to susceptible phenotypes obtained in our experiments, it is suggested that the recessive allelomorph of a gene in the nulliplex condition results in immunity, while the dominant allelomorph in the quadruplex condition gives complete susceptibility. The simplex and duplex conditions could give phenotypes with localized reactions, the one resulting from the former being the more efficient. Phenotypes reacting with severe systemic necrosis would be dependent on the triplex condition. There is no doubt that the operation of the major gene as suggested is tempered by polygenes.

VII. Acknowledgment

It is a pleasure to acknowledge the valuable technical assistance of Mr. J. W. Peak, Genetics Section of this Division, in this project.

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CARBONIC ANHYDRASE ACTIVITY IN PLANTS IN RELATION TO ZINC CONTENT

By J. G. Wood* and Pamela M. Sibly*

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Summary

Throughout the life cycle of oat plants carbonic anhydrase activity, which is restricted to the non-chloroplast fraction of leaves, reaches a maximum value and then decreases. After appearance of deficiency symptoms carbonic anhydrase activity is less in zinc-deficient plants than in fully manured controls of the same age and is associated with a lower zinc content of the leaves.

An experiment is described wherein uniform tomato plants were grown in zinc-deficient water cultures. The need for using uniform ontogenetic material is discussed. With the onset of zinc deficiency symptoms, which are described, four different concentrations of zinc were added to solutions in which the plants were growing; in order to minimize growth changes plants were harvested at short time intervals after application and carbonic anhydrase activity and zinc and protein-N contents were measured at each harvest.

Carbonic anhydrase activity showed highly significant positive linear correlation with both zinc and protein-N contents.

Evidence is presented which indicates that in zinc-deficient plants carbonic anhydrase activity is less than in normal plants through blocking of metabolic reactions leading to formation of protein and not by absence of sufficient zinc to activate an apoenzyme.

I. Introduction

Carbonic anhydrase (CA) activity in plants has been reported by Neish (1939), Day and Franklin (1946), Bradfield (1947), Waygood and Clendenning (1950), and Sibly and Wood (1951).

Keilin and Mann (1940) established that CA isolated from animal tissues was a zinc-protein enzyme.

The enzyme from plant sources differs from that present in animal tissues in several respects and notably in its relative insensitivity to metal-protein inhibitors. Day and Franklin (1947) found no inhibition of CA activity by cyanide in crude leaf extracts; Bradfield (1946) obtained about 70 per cent. inhibition of CA in crude extracts from sugar beet by 1.0×10^{-2}M KCN; using a partially purified enzyme preparation from spinach-beet, Sibly and Wood (1951) obtained 65 per cent. inhibition with 1.0×10^{-2}M KCN but practically no inhibition with 1.0×10^{-3}M KCN; and Waygood and Clendenning (1950) reported about 50 per cent. inhibition in crude extracts of *Tradescantia fluminensis* by

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1.0×10^{-3}M KCN, the percentage inhibition being greater at the same concentration of KCN for dialysed extracts. In crude extracts of tomato leaves, used in the work described below, 1.0×10^{-3}M KCN caused 50 per cent. inhibition and 1.0×10^{-2}M KCN complete inhibition of CA activity.

It is clear that preparations of CA from different species of plants vary appreciably in their sensitivity towards cyanide. Waygood and Clendenning (1950) have suggested that, if CA is a zinc protein, the zinc moiety may have less affinity for cyanide than other heavy metals present, and Sibly and Wood (1951) showed that copper as well as zinc was present in their preparations. However, all authors agree that CA preparations from plant sources are much less sensitive towards cyanide than those from animal sources; Keilin and Mann (1940) found that 4.0×10^{-6}M KCN caused 85 per cent. inhibition of the purified enzyme from animal tissues.

There is agreement among investigators concerning inhibition of CA activity from plant tissues by sodium azide; Bradfield (1947), Waygood and Clendenning (1950), and Sibly and Wood (1951) all report about 75 per cent. inhibition by 1.0×10^{-3}M azide.

The preparations of Keilin and Mann (1940) from blood contained 0.31-0.33 per cent. zinc; the purest preparations of Sibly and Wood contained 0.056 per cent. zinc, not removable by dialysis against water.

It cannot be said with certainty that CA in plants is a zinc protein but in this paper the relation between CA activity and zinc content of leaves has been investigated. Two experiments are described; in the first CA activity was measured at intervals throughout the life cycle of oat plants grown in water culture with and without addition of zinc; in the second tomato plants were grown in water cultures lacking zinc until incipient zinc deficiency symptoms appeared, zinc was then added to the culture solutions, and CA activity and zinc and protein-N contents of leaves measured at relatively short time intervals.

II. Carbonic Anhydrase Activity Throughout Life Cycle of Normal and Zinc-Deficient Oat Plants, Experiment 1

(*a*) *Material and Methods*

Wood and Sibly (1950) described the distribution of zinc in organs of oat plants (*Avena sativa* L. var. Mulga) at different growth stages. In the experiment described as experiment 3 in that paper, CA activity of leaves was also measured at each harvest, viz. 21, 49, 91, 121, and 140 days after sowing, and these data are now presented here. Details of cultural treatment and of solutions employed are described in the paper cited. Seeds were sown on May 17, 1948. The fully manured series (+Zn) received 0.2 mg. zinc per litre, the other series (—Zn) received no additional zinc. Lesions characteristic of zinc deficiency appeared on leaves of —Zn plants 95 days after sowing.

For estimation of CA activity, leaf extracts were prepared from aliquot samples of fresh material as described by Sibly and Wood (1951). For measurement of activity the "boat" apparatus of Meldrum and Roughton (1934)

was used and the unit of activity adopted was the "enzyme unit" (E.U.) of the same authors but measured at 5°C.

Zinc and copper contents were estimated polarographically by the method described by Wood and Sibly (1950).

Intact chloroplasts were isolated from aliquot samples of fresh leaf material and their chlorophyll contents determined by the methods described by Hanson, Barrien, and Wood (1941).

All measurements are expressed on a dry weight basis.

(*b*) *Results*

(i) *Localization of CA Activity.*—No CA activity was detected at any time in roots or stems, but only in leaves of oat plants. In a comparative study CA activity, zinc and chlorophyll contents were estimated in mature leaves of fully manured oats and spinach-beet and also in intact chloroplasts isolated from samples of these leaves. No CA activity was observed in chloroplasts of oats but with spinach-beet approximately 35 per cent. of the total CA activity in the leaf was localized in the chloroplasts. Since we carried out these estimations, Waygood and Clendenning (1950) have shown that in most plant species examined CA is adsorbed on chloroplasts and can be largely removed from them by repeated washings with water or with an alkaline buffer.

Table 1

CARBONIC ANHYDRASE ACTIVITY, ZINC AND COPPER CONTENTS OF OAT LEAVES AT DIFFERENT GROWTH STAGES, EXPERIMENT 1

Harvest	Time after Sowing (days)	Dry Wt./5 g. Fresh Wt. (g.)		CA Activity (E.U./g. dry wt.)		Zinc (μg./g. dry wt.)		Copper (μg./g. dry wt.)	
		+ Zn	— Zn	+ Zn	—Zn	+ Zn	— Zn	+ Zn	— Zn
1	21	0.443	0.443	104	104	76	76	28	28
2	49	0.540	0.545	111	110	276	87	75	61
3	91	0.565	0.627	120	80	119	27	26	23
4	121	0.770	0.780	81	41	41	11	15	13
5	140	0.840	1.100	50	34	19	7	7	11

In fully manured oats 18 per cent. of the total leaf zinc (9.7 μg Zn per g. fresh weight of leaves) and in fully manured spinach 52 per cent. of the total leaf zinc (2.9 μg. Zn per g. fresh weight of leaves) occurred in the chloroplast preparations. A partition of zinc between cytoplasm and its inclusions, probably variable in amount between species, therefore occurs, but since there is probably mobilization of all zinc reserves in deficiency the CA activity (apparently localized in the cytoplasm) has been related to the zinc content of the whole leaf.

(ii) *CA Activity in Leaves.*—CA activities and zinc and copper contents of leaves at each harvest in each series are set out in Table 1 and in Figure 1.

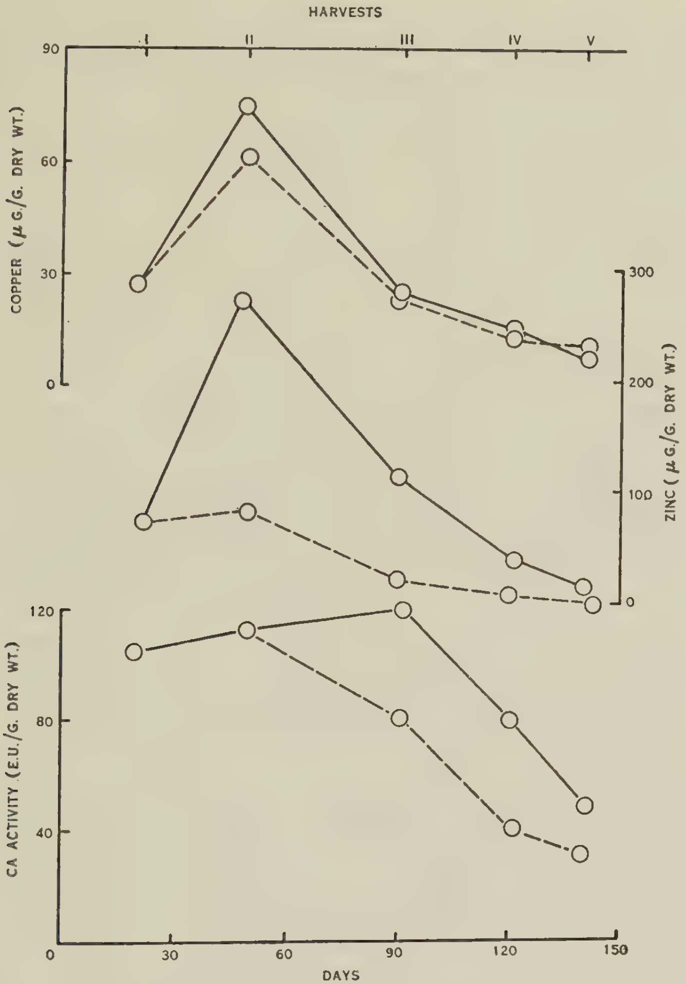

Fig. 1.—Drifts with time of CA activity, Zn and Cu contents in oat leaves, experiment 1. Solid line, + Zn plants; broken line, — Zn plants.

III. Effect of Added Zinc on CA Activity of Zinc-Deficient Tomato Plants, Experiment 2

Seeds of tomato (*Lycopersicon esculentum* Mill. var. Pan America) were soaked for 3 hours in cold bleaching powder solution and germinated on waxed mosquito netting over the culture solution described below. Seeds were sown on March 14, 1951. Sixteen days after sowing, seedlings were transferred to culture solutions in 3-litre Pyrex beakers covered externally with black paper.

Molar stock solutions were prepared and purified (Stout and Arnon 1939), and tested for heavy metal impurities with dithizone and chloroform. Glass-distilled water, tested similarly, was used to prepare all solutions. The composition of the culture solution was that recommended for tomatoes by Cheng Tsui (1948*a*). Each beaker contained 3 l. culture solution and plants were supported with paraffined cotton in holes drilled in waxed cane-board covers. The culture solution was aerated throughout each day.

Eight seedlings were placed in each of 10 beakers; the culture solutions in eight of these beakers received no zinc whilst that in the remaining two received 0.02 p.p.m. zinc as $ZnSO_4$.

Signs of zinc deficiency were first obvious 47 days after sowing when —Zn plants were smaller than controls with red-purple coloration at the base of the main stem. Between days 47 and 57 the following progressive symptoms appeared: reduction in size of plants was followed by reduction in size and number of leaves; yellow areas appeared on veins near the base of the leaves and spread outwards along the veins; later these chlorotic areas appeared in interveinal areas, causing a mottled appearance in the leaf; the leaves curled at the margins and leaf and leaflets became depressed owing to curvature of the petiole and midribs. At this time stems of —Zn plants were markedly purple, especially towards the base, and at a still later stage axillary buds developed to a greater extent in —Zn plants than in controls and these also were purple. On day 57 necrotic areas were beginning to dry out in a few leaves. On this day the mean height of plants was 14.9 cm. in —Zn plants and 23.5 cm. in controls; the mean number of leaves on —Zn plants and controls was 5.6 and 6.4 respectively, and the mean length of the third leaf above the cotyledons was 13.9 cm. on —Zn plants and 18.1 cm. on controls.

On day 58 the pots received the following treatments, two pots being used in each treatment: treatment *A*, original controls which had received 0.020 p.p.m. Zn 16 days after sowing; treatment *B*, no additional zinc; treatment *C*, 0.005 p.p.m. Zn; treatment *D*, 0.020 p.p.m. Zn; treatment *E*, 0.050 p.p.m. Zn.

Plants were harvested on three occasions, viz. harvest I, on day 58 immediately before application of zinc treatments; harvest II, 3 days after application of zinc treatments; harvest III, 7 days after application of zinc treatments.

At each harvest five plants were taken at random from each treatment, all leaflets removed and weighed. Duplicate samples, each of 2.5 g., were taken for immediate determination of CA activity. The remaining leaflets were dried and weighed, and zinc and protein N determined on replicate samples.

Leaves increased in dry weight during the experimental period, increment being least in treatment *B* (Table 2).

The dry weights of the 2.5 g. samples of fresh leaves used for CA estimation are given in Table 3. Analysis of variance performed on the data showed no significant effect of time or treatment. Over the time intervals used in our experiments addition of zinc did not cause significant increase in water content as described by Cheng Tsui (1948*b*). The material was homogeneous and

either fresh or dry weight would be equally satisfactory as a basis for expression of analytical data; a dry weight basis has been chosen.

TABLE 2

MEAN DRY WEIGHTS OF LEAVES PER PLANT (G.) AT EACH HARVEST, EXPERIMENT 2

Treatment	Harvest I	Harvest II	Harvest III
A	0.96	1.06	1.36
B	0.41	0.37	0.50
C	0.49	0.59	0.71
D	0.58	0.65	0.76
E	0.49	0.68	0.57

CA activity and zinc content of leaves were estimated as described in the previous experiment. For determination of protein N, aliquots of the finely ground leaf material were warmed at 40°C. for 15 minutes with distilled water, boiled for 1 minute, cooled, the pH adjusted to 4.5 with trichloracetic acid, and filtered; nitrogen was determined on the residue by the micro-Kjeldahl method. Mean values for all estimations are presented in Table 4.

TABLE 3

DRY WEIGHTS (G.) OF THE 2.5 G. SAMPLES OF FRESH LEAVES USED FOR CA ESTIMATIONS, EXPERIMENT 2

Treatment	Harvest I	Harvest II	Harvest III
A	0.230	0.229	0.258
B	0.228	0.183	0.236
C	0.227	0.224	0.206
D	0.236	0.239	0.210
E	0.230	0.240	0.203

IV. DISCUSSION

Data from the experiment with oats (Fig. 1) show that CA activity on a dry weight basis increases to a maximum value and then decreases throughout the latter part of the life cycle, although maximum values do not occur at the same time in normal and in deficient plants.

A similar temporal drift in most leaf constituents on a dry weight basis, e.g. nitrogen, phosphorus, and inorganic constituents generally (cf. Petrie 1937; Goodall and Gregory 1947; Piper and Walkley 1943), is a general feature for leaves analysed at different stages in their life cycle. The chief causes of these drifts are exponential increase in amount of dry matter at a time when external

supply of nutrients is decreasing and translocation of materials occurs from leaves to other developing organs.

The temporal drifts of zinc and copper contents together with CA activity are shown in Table 1 and Figure 1. Trends for zinc and copper contents are similar and follow the usual pattern, but it is clear that, whilst the amounts of copper are approximately the same at each harvest in both + Zn and — Zn plants, the amounts of zinc are considerably less in — Zn plants than in controls.

Table 4

MEAN VALUES OF CA ACTIVITY, ZINC AND PROTEIN-N CONTENTS OF TOMATO LEAVES, EXPERIMENT 2

Treatment	Harvest	CA Activity (E.U./g. dry wt.)	Zinc (μg/g. dry wt.)	Protein N (μg/g. dry wt.)	Ratio (Zn/CA)	Ratio (Protein N/CA)
A	I	303	69	36.7	2.28	1.21
(control)	II	317	75	33.2	2.27	1.05
	III	315	72	29.2	2.28	0.93
B	I	62	21	18.0	3.39	2.90
(Zn nil)	II	69	34	18.1	4.93	2.63
	III	62	37	18.6	5.97	3.00
C	I	98	51	23.2	5.20	2.37
(Zn 0.005 p.p.m.)	II	110	98	25.6	8.91	2.33
	III	118	91	27.9	7.72	2.36
D	I	72	28	22.8	3.89	3.16
(Zn 0.020 p.p.m.)	II	79	65	21.5	8.85	2.72
	III	115	97	29.2	8.44	2.54
E	I	69	22	21.6	3.19	3.13
(Zn 0.050 p.p.m.)	II	81	37	22.1	4.57	2.73
	III	96	65	27.9	6.77	2.90

In the — Zn series CA activity follows the same trend as does zinc content; for + Zn plants this is only true during the latter stages of the life cycle. During early stages of the life cycle CA activity in + Zn plants shows small increase associated with large increase in zinc content between harvests I and II, and between harvests II and III CA activity increased whilst zinc content decreased.

It is not legitimate to compare relations between CA activity and zinc content at different harvests since the plants are in different physiological states and stages of ontogeny, and the basis of expression alters markedly with time (cf. dry weights per 5 g. fresh weight at each harvest in Table 1). In this experiment comparisons can be made between CA activity and zinc content at the same harvest in the two series and it is apparent that at the last three harvests (i.e. after appearance of zinc deficiency symptoms in — Zn plants) the CA activity of — Zn plants is less than that of the controls and is associated with low zinc content of — Zn plants compared with controls.

Similarly in the experiment with tomatoes, + Zn plants (treatment *A*, Table 4) possessed appreciably higher CA activity than — Zn plants.

There is evidence therefore that, compared with that in controls, CA activity is relatively low in zinc-deficient plants, but the type of experiment described above does not permit relations between CA activity and zinc content to be established.

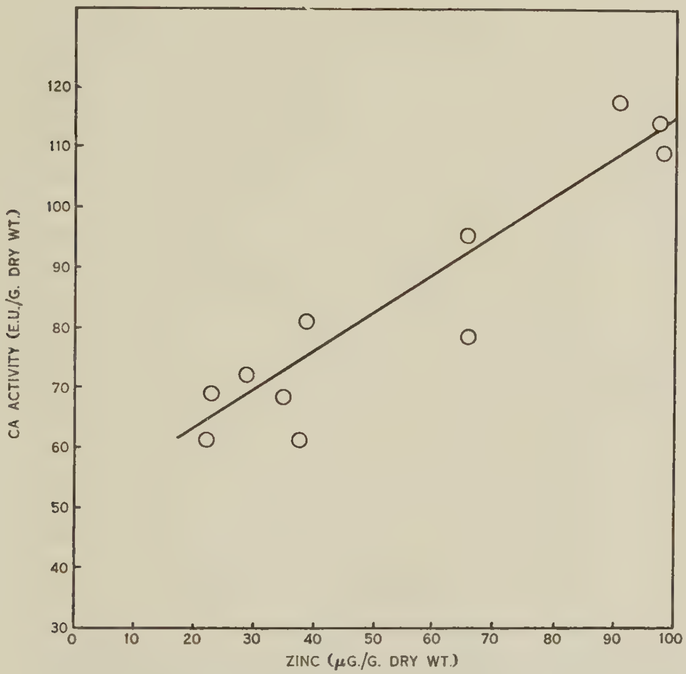

Fig. 2.—CA activity plotted against Zn content of tomato leaves, experiment 2. The line is that of the equation CA = 50.74 + 0.654 Zn; the coefficient of Zn is significant at $P < 0.01$.

If CA in plants is a zinc-protein enzyme, the effect of zinc deficiency in decreasing CA activity observed in the previous experiment might be due to one of the following possibilities:

(*a*) Zinc may not be present in amount sufficient to combine with all the apoenzyme present to form CA.

(*b*) Zinc deficiency may limit other metabolic processes, e.g. one of the precursors of the protein moiety of the enzyme.

To decide between these possibilities, the experimental technique adopted was to grow tomato plants in culture solutions lacking in zinc and so provide uniform ontogenetic material; varying amounts of zinc were then added to the culture solutions in which these uniform plants were growing and, after short time intervals to minimize growth changes, CA activity and zinc and protein-N contents were determined.

In seeking for relations between CA activity and the other constituents measured the whole of the data from treatments *B*, *C*, *D*, and *E* have been pooled. Plants of treatment *A* were at a different growth stage and cannot be compared with the foregoing.

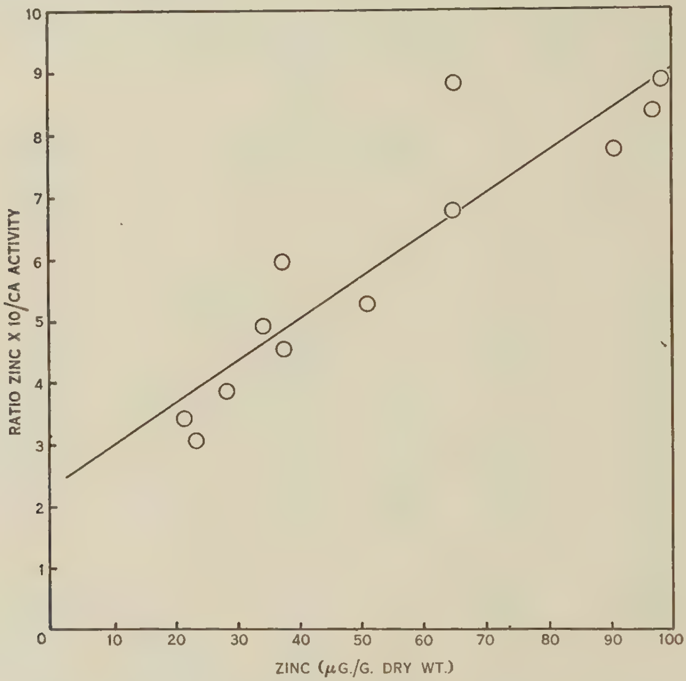

Fig. 3.—Ratio Zn × 10/CA activity plotted against Zn content, experiment 2. The line is that of the equation Zn × 10/CA = 2.37 + 0.067 Zn; the coefficient of Zn is significant at $P < 0.01$.

CA activity shows highly significant correlation ($P < 0.01$) with zinc content (see Fig. 2). If CA is a zinc-protein enzyme and if zinc were utilized as the prosthetic group and not for other tissue processes to the same extent, then the ratio zinc/CA activity should tend to remain constant as the zinc content increases until the apoenzyme was fully activated, when the ratio should increase. In Figure 3 the ratio zinc × 10/CA activity is plotted against zinc concentration. No such relation as that postulated is evident; the ratio increases in a linear manner with increased zinc content. It is apparent that zinc did not limit CA activity in zinc-deficient plants by its presence in concentration too low to activate a ready-formed apoenzyme.

The second alternative stated above was therefore investigated, viz. that zinc could affect CA activity by limiting the formation of the protein part of the enzyme. From Figure 4 it can be seen that protein-N content of the leaves of the experimental plants shows a highly significant positive correlation ($P < 0.01$) with zinc content. Figure 5 shows a highly significant positive cor-

relation ($P < 0.01$) between CA activity and protein N. The ratio protein $N \times 10/CA$ activity plotted against protein N (Fig. 6) is approximately constant over the whole range of values of protein N.

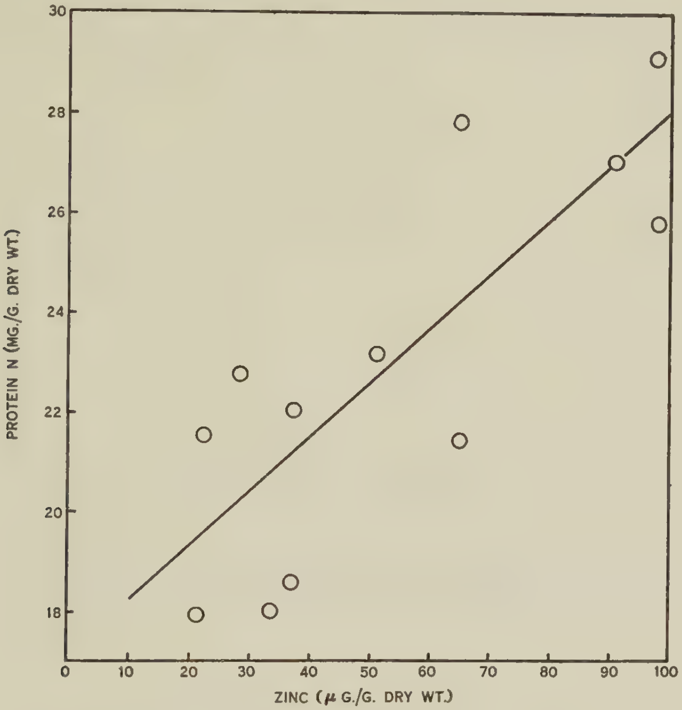

Fig. 4.—Protein N plotted against Zn content, experiment 2. The line is that of the equation protein $N = 17.17 + 0.109$ Zn; the coefficient of Zn is significant at $P < 0.01$.

We conclude therefore that deficiency of zinc limits the formation of CA as well as that of other proteins. The experiments described here throw no light on the problem whether CA is a zinc protein; if it is one, then the concentration of zinc required for formation of the protein moiety is higher than that required for the prosthetic group.

The results described in this paper draw attention to two points which should be mentioned. First, the need for care in comparing enzyme activities in deficient and normal plants should be emphasized. It is apparent that zinc deficiency causes decrease in CA activity; it is probable that deficiency of any inorganic nutrient which is essential for any of the stages antecedent to protein formation would also cause decreased CA activity. Second, plants with different ontogenetic and cultural history should not be compared. It should be noted in Table 4 that the values in any category for the fully manured series (treatment *A*) belong to a different population of values from those in the experimental series; in particular CA as a percentage of the total protein is higher in treatment *A* than in the experimental series.

Our finding that zinc is essential for production of CA in particular and for protein in general supports the results of Cheng Tsui (1948*a*) who con-

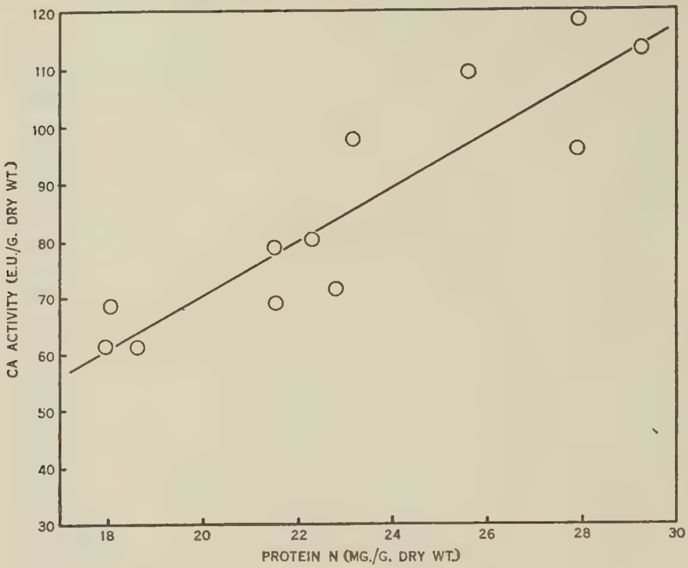

Fig. 5.—CA activity plotted against protein-N content, experiment 2. The line is that of the equation CA = — 23.5 + 4.75 protein N; the coefficient of protein N is significant at $P < 0.01$.

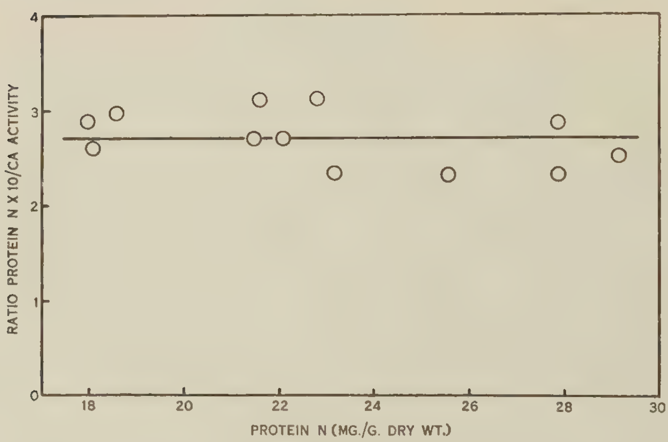

Fig. 6.—Ratio protein N × 10/CA activity plotted against protein-N content, experiment 2. The line is that of the mean value of protein N/CA, 2.73.

cluded that the decreased auxin activity observed in zinc-deficient plants was associated with decreased tryptophane content; addition of zinc resulted in increased tryptophane and auxin contents.

Quinlan-Watson (1951) has shown that aldolase activity is greatly decreased in zinc-deficient plants. This suggests that zinc deficiency restricts protein formation through limiting the metabolism of hexose diphosphate. This may be so but cannot yet be accepted as conclusive. As pointed out above, zinc deficiency by limiting protein formation is likely to decrease the activity of any enzyme compared with that of the fully manured controls.

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THE EFFECT OF GLUCOSE IN THE GROWTH MEDIUM ON THE SUCCINIC ACID OXIDIZING SYSTEM OF *ESCHERICHIA COLI*

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Summary

Manometric determinations have been made of the rate of succinate oxidation by washed *Escherichia coli* cells derived from a glucose-containing medium. Succinate oxidation is initially slow but after 50-60 minutes the rate increases to a steady level. The duration of the lag period is markedly increased by excessive washing of the cells. With minimally washed cells the final activity may be reduced as a result of competitive inhibition due to endogenous substrates. Conditions of suspension preparation must be carefully standardized to obtain maximum activities and reproducible effects. Results obtained indicate that the enzyme system responsible for the oxidation of succinate is formed when *Escherichia coli* is grown in a glucose medium and that the lag period can be more satisfactorily explained on the basis of a temporary impermeability of the cell to succinate rather than a period of enzyme adaptation.

I. Introduction

In his review on factors influencing the enzymic activities of bacteria, Gale (1943) points out that the formation of many bacterial dehydrogenases is inhibited when glucose is included in the growth medium. He also states that although anaerobic conditions are unfavourable for dehydrogenase production, the inhibition observed when cells are grown in a glucose medium is due neither to anaerobiosis nor to acid production during growth. Happold and Hoyle (1936) and Evans, Handley, and Happold (1942) have shown with the tryptophane oxidizing system of *Escherichia coli* that no indole is produced in glucose-containing media. Washed suspensions of these glucose-grown cells show no initial ability to produce indole from tryptophane but do so after a definite lag period. Dawson and Happold (1942) have shown that the lag period is associated with the breakdown of stored carbohydrate. Gale has suggested in his review that enzyme variations observed with glucose-grown cultures may be due to alterations in the permeability of the cell membrane under the influence of polysaccharide deposition.

The purpose of the present investigation has been to study the effect of glucose in the growth medium on the formation of the succinic acid oxidizing system of *Escherichia coli*.

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II. Materials and Methods

A laboratory strain of *Escherichia coli* type I was used. Stock cultures were maintained on yeast extract agar prepared by solidifying yeast extract broth ("Difco" peptone 1.0 per cent., "Difco" yeast extract 0.5 per cent., and sodium chloride 0.5 per cent.) with "Davis" agar 1 per cent. In all experiments the organisms were propagated by inoculating 100 ml. of yeast extract broth contained in 250-ml. conical flasks and incubating at 37°C. for 18 hours as standing cultures. Harvesting and washing of cells, using M/15 phosphate buffer (pH 7.5), was carried out at 5°C. at 3000 r.p.m. (4100g).

The oxidation of succinate was followed in Warburg manometers, the contents of the cups being as follows: M/15 phosphate buffer (pH 7.5) 1.5 ml.; cell suspension 1.0 ml.; M/50 sodium succinate 0.3 ml. (6μM); distilled water to 3.0 ml. Potassium hydroxide (10 per cent.) was used in the centre well in all experiments.

Nitrogen determinations were carried out on all cell suspensions by digesting in a mixture of sulphuric acid, copper sulphate, and selenium oxide, followed by nesslerization.

III. Experimental

(*a*) *Comparison of Cells Grown in the Presence and Absence of Glucose*

An initial experiment was carried out to compare the rate of succinate oxidation by cells grown in yeast extract broth to that of cells grown in yeast extract broth containing 1 per cent. glucose. The cultures were grown in 250-ml. conical flasks, each containing 100 ml. medium, and were harvested and washed as described above. The results are shown in Figure 1. Whereas the rate of oxygen uptake for the non-glucose-grown cells proceeds in a linear fashion from zero time to completion, the rate for the glucose-grown cells is initially very slow but after about 50-60 minutes there is a marked increase, after which oxidation proceeds steadily until all the succinate has been oxidized. The total amount of oxygen utilized in the oxidation of 6μM of succinate is about 330 μl., i.e. approximately 2.5 moles of oxygen per mole of succinate. Hence it appears that there is an oxidative assimilation of some of the succinate as 3.5 moles of oxygen are required for the complete oxidation of one mole of succinate to carbon dioxide and water.

(*b*) *Effect of Washing the Cells*

The curve for succinate oxidation shown in Figure 1 for glucose-grown cells was found to be difficult to reproduce and the following experiment demonstrates that the technique used for washing these cells greatly affects their rate of oxidation of succinate. An 800-ml. glucose broth culture was prepared and divided into 200-ml. aliquots. The deposit obtained after centrifuging the first aliquot (*A*) was washed once with 20 ml. of buffer, the second (*B*) was washed three times with 20 ml. each time (total 60 ml.), the third (*C*) five times (100 ml.), and the fourth (*D*) seven times (140 ml.). After

washing, each batch of cells was suspended in 5 ml. of buffer. These operations were carried out as quantitatively as possible and the final cell suspensions contained 1.3 ± 0.1 mg. cell N/ml. Each suspension was tested for its ability to oxidize succinate and the results are given in Figure 2. As the amount of washing is increased, the length of the lag period is increased and the final activity becomes progressively less. Addition of boiled suspension *A* to suspension *D* did not show any significant increase in final activity or decrease in the lag time. Shown also in Figure 2 are results obtained by using non-glucose-grown cells which had been washed with 20 ml. and 140 ml. of buffer. Washing has no effect on the succinate oxidizing system of these cells.

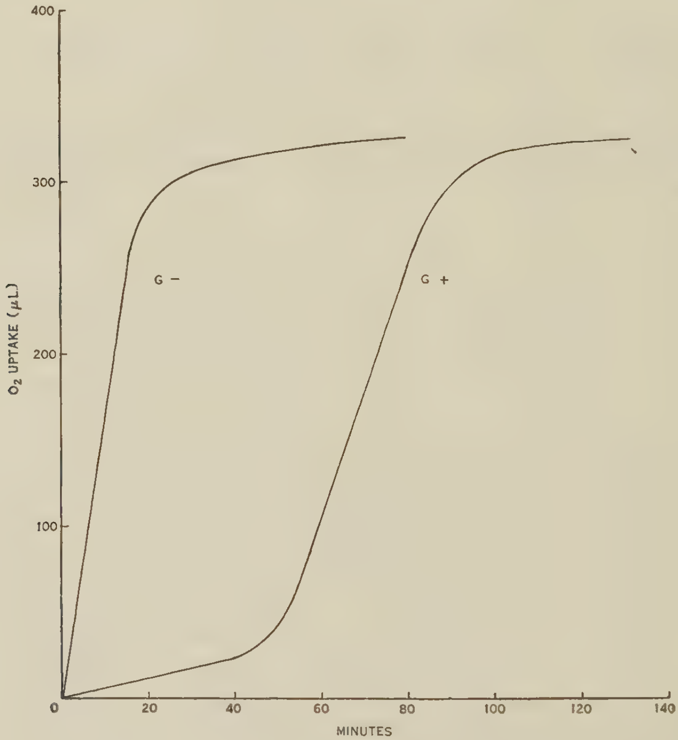

Fig. 1.—Comparison of oxygen uptake rates of *Esch. coli* cells grown in glucose broth (G+) and broth without glucose (G—). Succinate: 6μM per cup. Cell suspensions 1.2 mg. N/ml. Oxidation rates have been corrected for endogenous respiration in all figures.

(*c*) *Effect of Endogenous Respiration*

It would be expected from the results shown in Figure 2 that the best final activity would be obtained if the cells were given minimum washing. However, if glucose-grown cells from 200 ml. of culture are washed with only 10 ml. of buffer it is found that, after correction for endogenous metabolism (which

is naturally high) the activity is less than anticipated. The only difference between this experiment and that yielding curve *A* in Figure 2 is that there is present a higher concentration of endogenous substrates. The decreased activity associated with a high rate of endogenous respiration is well illustrated if the effect of varying the concentration of minimally washed glucose-grown cells in the presence of a constant amount of succinate (6μM) is determined. Reference to Figure 3 will show that a linear relationship holds for

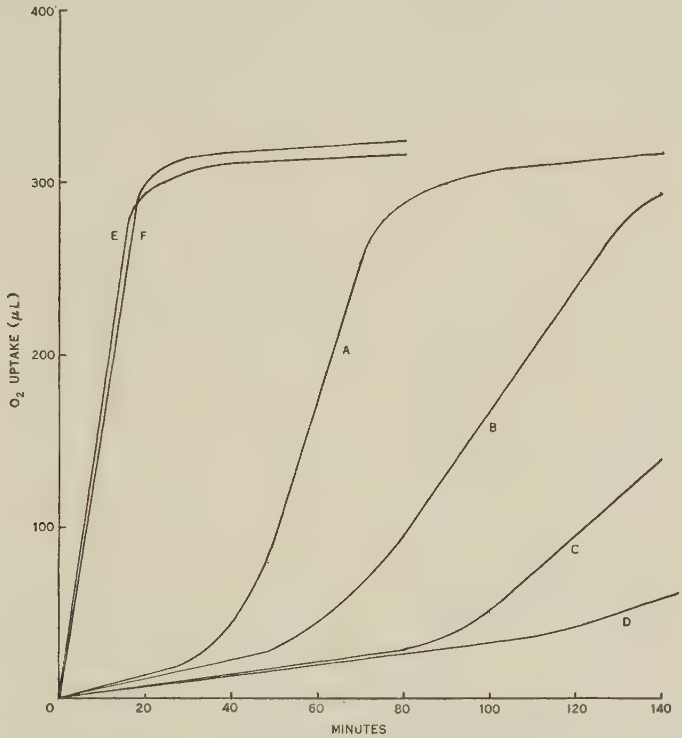

Fig. 2.—Effect of washing cells of *Esch. coli* derived from glucose broth (*A, B, C,* and *D*) and broth without glucose (*E* and *F*). *A* and *E* were washed with 20 ml. of buffer, *B* with 60 ml., *C* with 100 ml., and *D* and *F* with 140 ml. Succinate: 6μM per cup. Cell suspensions 1.3 mg. N/ml.

a limited range of cell concentrations only. At high concentrations the relative, and finally the total, activity decreases. The activities of the suspensions in this experiment are corrected for endogenous respiration and represent the maximum rate of oxidation after the lag period. For non-glucose-grown cells a linear relationship holds for suspension concentrations of 0-2.0 mg. N/ml. These results indicate that when a high rate of endogenous respiration is associated with the cell suspension there is a competitive inhibition of succinate oxidation by the endogenous substrates. That the rate of oxidation by heavier suspensions of minimally washed cells is due to such a competitive inhibition

was confirmed by increasing the concentration of succinate. The results are given in Figure 4 and show that a fivefold increase in succinate concentration largely overcomes this inhibition.

In the light of this information it is clear that, in order to measure the activity of a suspension when the usual concentration (6μM) per cup) of succinate is used, the cell concentration must be that which gives an activity that falls on the linear portion of the curve in Figure 3. It will be appreciated, of course, that the linear portion of the curve may be extended by a limited increase in the amount of washing.

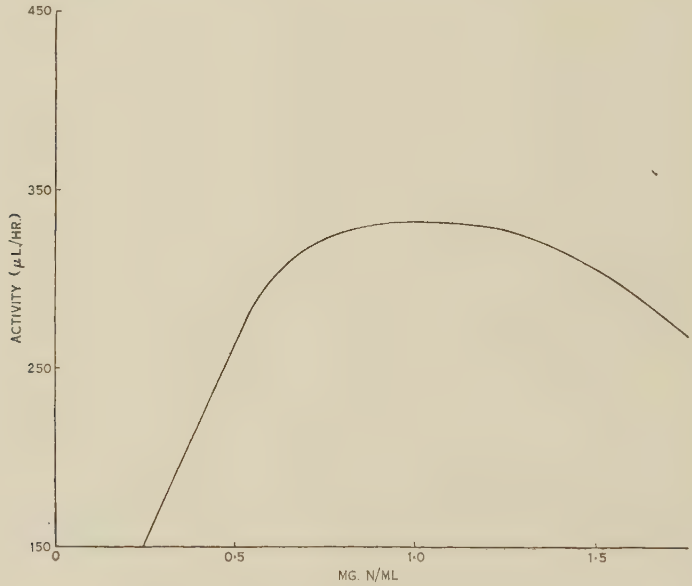

Fig. 3.—Effect of varying the concentration of minimally washed glucose-grown cells on the rate of oxidation of succinate. Succinate: 6μM per cup.

A procedure for suspension preparation which satisfies the above conditions and has been found to give uniform results is as follows: The centrifuged deposit from the broth culture is suspended in M/15 phosphate buffer (pH 7.5) to give an optical density of 0.969 in the Evelyn colorimeter using a 660 filter (about 0.4 mg. cell N/ml.). Ten ml. of this suspension are centrifuged and suspended in 3.5 ml. of buffer (final cell N = 1.0 mg./ml.).

(*d*) *Observations on the Lag Period*

There are two possible explanations for the lag period. The first assumes a temporary inability of the cells to oxidize succinate and the second suggests that it is a period of enzyme adaptation. If the first explanation is correct, and the temporary inability to oxidize succinate is due to the presence of a meta-

bolizable polysaccharide then incubation of the cells for a period before the succinate is added would remove the lag period. Should the second explanation hold, then such pre-incubation would not remove the lag period. The experiment was carried out in the following manner. Five manometer flasks were set up in the usual way, four with succinate in the side-arms and a control. After equilibration the substrates were tipped in at zero time, 15, 40, and 70 minutes. Reference to Figure 5 will show that the lag periods, as determined from the time of substrate addition, become progressively less as the pre-incubation time is increased. However, even after 70 minutes pre-incubation a small (5-10 minutes) lag period remained. It was also found that the lag period could be markedly reduced by bubbling oxygen through the suspension for half an hour at 37°C. before testing.

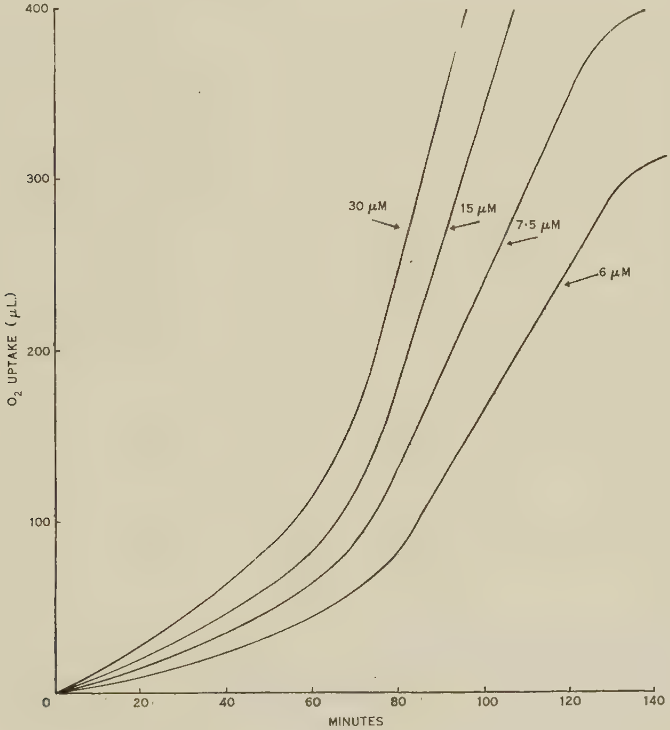

Fig. 4.—Effect of varying the succinate concentration on the activity of minimally washed glucose-grown cells. Cell concentration: 2.0 mg. N/ml.

In order to obtain perfectly uniform results it was essential to allot standard times for all operations from the moment the cells were removed from the refrigerated centrifuge until the substrate was added after equilibration.

IV. Discussion

A reinvestigation of the formation of the succinic acid oxidizing system of *Escherichia coli* when grown in the presence of glucose has shown that the

system is formed and that the oxidation of succinate proceeds rapidly after a preliminary lag period. With cells thoroughly washed in the usual way the lag period is so markedly prolonged and the final activity decreased that the presence of the system could quite easily be missed if the normal experimental time limits are used. The factor or factors affecting the activity which are removed by washing are apparently heat-labile as the addition of a boiled minimally washed suspension to a thoroughly washed suspension does not enhance the activity of the latter.

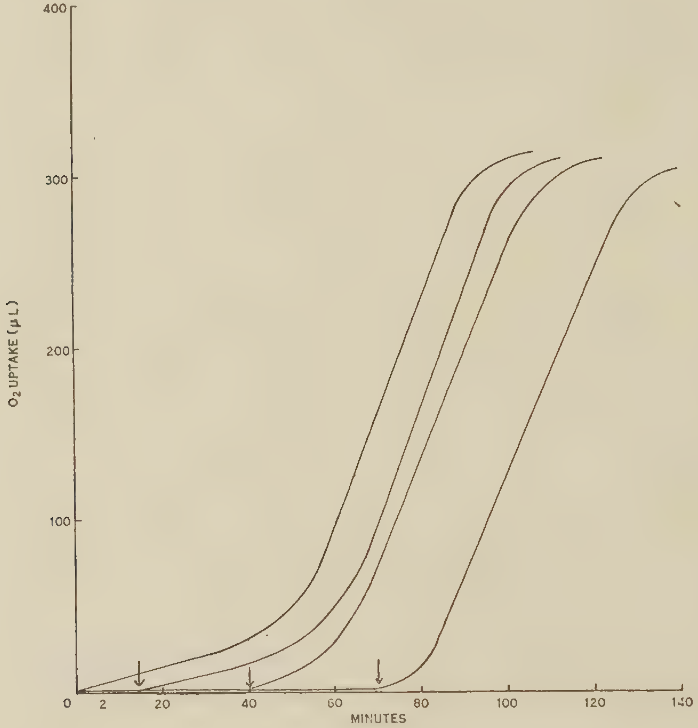

Fig. 5.—Effect of pre-incubation of the manometer cup contents before the addition of succinate. Manometers were tipped at 0, 15, 40, and 70 minutes. Succinate: 6μM/cup. Cell suspension: 1.0 mg. N/ml.

Results presented in this paper indicate that the lag period observed is not due to enzyme adaptation (cf. Stanier 1947) for two reasons. Firstly, most of the lag period can be removed by pre-incubation of the suspension in the absence of the substrate, or by aeration, and secondly, well-washed suspensions show a much increased lag period. Generally, the results indicate that the cell is temporarily unable to oxidize succinate and this may be due, as suggested by Evans, Handley, and Happold (1942) for "tryptophanase," to the production during growth of a metabolizable polysaccharide which renders the cell temporarily impermeable to the substrate.

Ajl (1950), in applying the technique of simultaneous adaptation to the breakdown of glucose and acetate by *Escherichia coli,* found that glucose-grown cells showed no lag period in their oxidation of succinic acid using cells which "were often aerated for 2-3 hours in phosphate buffer to reduce endogenous metabolism." It is possible that there would have been a lag period had the cells been treated as described in this paper. Therefore in simultaneous adaptation experiments the nature of the curves obtained and the conclusions drawn from them may become a function of the technique of suspension preparation. Campbell and Stokes (1951) have shown that by using washed cells a lag period was obtained with several substrates tested, including succinate, but that no lag period was found if the cells were dried before testing. They concluded that the lag periods observed may be due more to permeability effects than to enzyme adaptation.

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OVIPOSITION AND MATING BEHAVIOUR OF THE QUEENSLAND FRUIT-FLY (*DACUS* (*STRUMETA*) *TRYONI* (FROGG.)) AND THE SOLANUM FRUIT-FLY (*DACUS* (*STRUMETA*) *CACUMINATUS* (HERING))

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Summary

A method for the laboratory culture of the Queensland fruit-fly (*Dacus tryoni* (Frogg.)) is described.

Evidence is presented to show that larval production in *D. tryoni* is influenced by the intensity of light experienced during the day. In apples under the higher intensities of artificial light more larvae are produced than under lower intensities of daylight alone.

It has been shown that the lower light intensities prior to darkness are necessary for the mating activity of *D. tryoni*. Under natural conditions mating behaviour is stimulated by these lower intensities. No mating normally occurs when this dusk period is omitted from the daily light conditions to which the insects are exposed.

Light intensity throughout the day has been shown to affect the rate of male mating activity in the evening, using copulatory activity and male mating 'calls' as criteria.

Evidence is presented to show that *D. tryoni* and the solanum fruit-fly (*D. cacuminatus* (Hering)) do not interbreed and an introductory analysis of the barriers to crossing is made.

I. INTRODUCTION

The insect with which this paper deals principally (*Dacus tryoni*) is a pest species of fruit-fly belonging to the family Trypetidae and inhabits the humid area of eastern Australia.

In common with most other trypetids, the female penetrates the skin of the fruit by means of a sharp, sting-like ovipositor and deposits eggs in a small oviposition chamber. Upon hatching, the larvae feed on and penetrate the fleshy tissue of the fruit. The fruit falls to the ground and the larvae migrate into the soil to pupate and emerge later as adults.

General ecological notes covering the life cycle and other features of the biology of the Queensland fruit-fly and other Australian trypetids have been published by Gurney (1912), Wright (1937), Allman (1938, 1940, 1941), Perkins (1934, 1937, 1938), and Perkins and May (1949).

The breeding of Australian Trypetidae under controlled conditions has not yet been recorded, although Allman (1938) bred *D. tryoni* successfully under uncontrolled room conditions. Attempts to repeat his breeding experiments in

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this laboratory were unsuccessful. Numerous workers in America have described common difficulties in breeding different species of Trypetidae in the laboratory. O'Kane (1914) and Potter (1928) found that egg development was either slow or absent. Illingworth (1912), Lathrop and Nickels (1931), Boyce (1934), McAlister and Anderson (1935), and others all reported abnormal breeding behaviour under laboratory conditions. In many cases no offspring were produced. Where eggs were laid the numbers fell far short of those oviposited in the field.

From studies such as these it would appear that careful techniques will have to be developed before this insect can be studied in the laboratory. The work carried out in this paper was designed primarily to develop a method for the laboratory culture of *D. tryoni*. During preliminary work, observations suggested that light intensity influenced fecundity, and evidence is presented that this is so. In addition, the importance of the changing light intensity prior to darkness is shown to be necessary for successful mating activity.

Concomitantly with the above work, numerous observations on the mating behaviour of *D. tryoni* were made. Since this had not previously been described a short account has been included. Attempts to cross the Queensland fruit-fly and the solanum fruit-fly (*D. cacuminatus*) were also made.

The names of the two species under study were taken from a recent and comprehensive paper on the taxonomy of Australian Trypetidae (Hardy 1951).

II. Influence of Light Intensity on the Larval Production of D. tryoni

(*a*) *Materials and Methods*

The insects used were laboratory-bred stock raised under the breeding conditions outlined below. The initial population was obtained as larvae in oranges from the Castle Hill district, N.S.W.

The room used measured 10 by 7 by 9 ft. high, with a large window, 6 by 4 ft. at the southern end. This window was covered internally by clear celluloid to aid the insulation of the room. A temperature of 22°-24.5°C. and a relative humidity of 62-68 per cent. were maintained throughout the cooler months of the year.

Experimental cages were of flint glass, measuring 8 by 3 by 12 in., each with a sleeve of fine muslin fastened about the rim of its open end (8 by 3 in.) by means of sticking plaster. Each cage was washed at least once a week. A strip of dark paper was glued to the inner surface of the ceiling of each cage to provide a dark resting place for the flies.

The food finally selected was made from the following formula, slightly modified from one recommended by Dr. G. B. Mainland (personal communication), University of Hawaii.

Paw paw paste	600 ml.
Banana paste	100 ml.
Peeled orange	60 ml.
Honey	10 ml.

The mixture was placed daily into each cage on a small watch-glass or glass slide which was washed daily.

Water was supplied in small jars with cotton-wool plugs. The cotton-wool remained damp for many days but it was found necessary to replace the plugs frequently owing to a slimy covering apparently derived from the regurgitations of the insects.

The breeding population used was six pairs of flies to each cage or one fly per 30 cu. in. Five cages were placed at a time around the circumference of a circle of radius 12 in. about a 150-watt clear Mazda lamp, the base of which was plugged into a socket 1 in. above the surface of the table used. Each cage was arranged so that one of the 12 by 8 in. sides faced the light directly. Owing to the proximity of the lamp, a rise of 1°C. above the average room temperature occurred within each cage.

In the spring and summer, 7½ hours of electric light were given daily from 9.30 a.m. to 5.00 p.m. In the autumn and winter the times were altered to 9.00 a.m. and 4.30 p.m. This was found necessary to give the breeding populations a dusk period to stimulate breeding activity. In winter, the room became dark very early. Prior to dusk, the cages were carried to a table under the window.

Owing to the relatively low daily oviposition rate, counts of larvae were taken on a weekly basis. One well-washed apple was placed in each cage every Monday and was replaced by a second each Thursday. This procedure ensured that eggs of widely differing ages were not oviposited in any apple.

To prevent competition for oviposition sites a circle of 30-40 pin-pricks (Allman 1938) was made in each apple immediately before it was placed in the cage. Until the apple was removed from the cage a fresh circle of punctures was made each day, since this appeared to stimulate oviposition.

It was not possible to make accurate egg counts in apples. Against the white flesh of the fruit, eggs and young larvae were not easily seen. Consequently larval counts were made after the larvae had reached a rather advanced stage of development. This technique thus did not take into account infertile eggs or larval mortality, which together may have reached considerable proportions. The use of tomatoes as oviposition sites afforded a better method of counting eggs but the quick decomposition of the fruit under warm-room conditions made this impracticable.

Whenever fresh adults for experimental purposes were needed, apples from stock cages were placed on sand within a cage of mesh fine enough to prevent the entry of *Drosophila*. The pupae were then either sieved or carefully washed from the sand and placed into small flat-bottomed tubes where they were again covered with sand. The tubes were then plugged with cotton-wool. Upon emergence, the adults were sexed and released into the required cage.

(*b*) *Durations of Immature Stages*

Under the above conditions the different stages in development were found to require the following periods of time.

Embryonic development.—Of 90 eggs squeezed from tomatoes, 93 per cent. hatched in 43 ± 2 hours.

Egg to pupation.—Counts taken from the day the eggs were laid to the date of pupation of the larvae developing from those eggs showed a maximum on the thirteenth day. These counts are graphed in Figure 1.

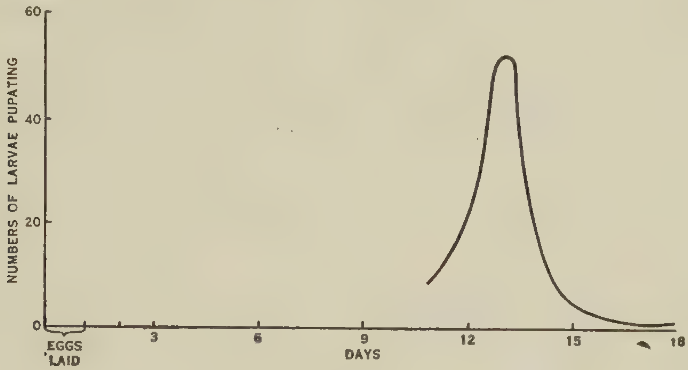

Fig. 1.—Duration of development of the combined egg and larval stages of *D. tryoni.*

Pupal stage.—From pupae collected on a known date, adults were found to emerge mainly on the thirteenth day. The counts of a sample of 121 are graphed in Figure 2. The full life cycle from egg to egg under these condi-

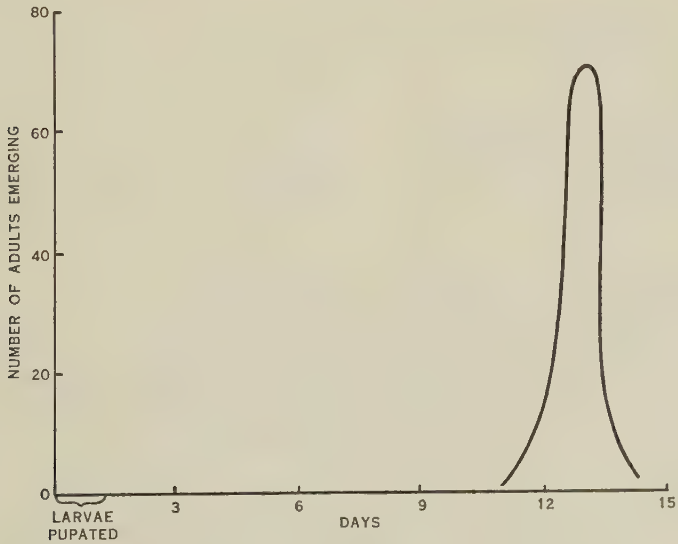

Fig. 2.—Length of pupal development of *D. tryoni.*

tions averaged 7 weeks, including a pre-oviposition period of 14-21 days. It is not known whether this long pre-oviposition period is due to inadequacies in technique or is characteristic of the species.

TABLE 1

INFLUENCE OF LIGHT INTENSITY ON THE NUMBER OF LARVAE FOUND IN APPLES

	Cage	Day Mating Activity Commenced	Day Copulation Observed	Day First Fertile Eggs Laid	Number of Larvae During Week 1	2	3	4	5	6	7
Breeding population exposed to 150-watt lamp	A1	11	16	21	15	20	28	47	24		
	A2	9	9	14	7	15	31	57	59		
	A3	18	20	21	8	21	72	68	28		
	A4	13	14	15	4	41	99	102	53		
	A5	14	15	16	6	44	77	78	55		
	Mean	13	14.8	17.4	8	28.2	61.4	72.4	43.8		
	St. dev.	3.8	3.96	3.3		13.16	25.58	21	16.8		
	Mean/fem.				1.3	4.7	10.23	12.07	7.3	2.5 (13 ♀ ♀)	1.15 (9 ♀ ♀)
Breeding population exposed to diffused daylight	B1	20	22	23	3	1	4	25	5		
	B2	21	22	24	1	8	8	22	18		
	B3	16	16	22	8	11	9	34	31		
	B4	15	15	18	1	16	28	52	33		
	B5	16	16	18	1	6	24	33	24		
	Mean	17.6	18.2	21	2.8	8.4	14.6	33.2	21.2		
	St. dev.	2.8	3.5	3.0		5.8	10.7	11.9	11.3		
	Mean/fem.				0.47	1.4	2.43	5.53	3.53	2.1 (19)	1.3 (13)
Adult population maintained in complete darkness	C1				0	0	0	0	0		
	C2				0	0	0	0	0		
	C3				0	0	0	0	0		
	C4				0	0	0	0	0		
	C5				0	0	0	0	0		
	Mean/fem.				0	0	0	0	0		
Significance of differences between weekly means of series A and B, using 't' test						$P = 0.02$	$P = 0.01$	$P = 0.01$	$P = 0.02$		

In series C the weekly counts represent each week of life. An 'F' test showed no significant differences at the 0.05 level between the variances of corresponding weekly counts in series A and B.

(*c*) *Experimental Design*

Three series were set up, each of five cages, with six pairs of flies per cage, under the following conditions:

Series *A*.—Exposed for 7½ hours daily to the 150-watt lamp (9.30 a.m. to 5.00 p.m.). These cages received in addition subdued daylight through the window. From 9.00 a.m. to 3.00 p.m. on a bright day (February) the light intensity measured by a Weston Master exposure meter (Model S.74/715) at the face of each cage was found to be 200 candles per sq. ft.

Series *B*.—Shielded from the electric lamp and exposed to subdued daylight alone. The light intensity measured as in series *A* amounted to 25 candles per sq. ft.

Series *C*.—Maintained under conditions of complete darkness.

(*d*) *Results*

The observations (Table 1) together with the graph of weekly larval production (Fig. 3) provide a certain amount of evidence to support the theory that daily light intensity affects fecundity. Series *C* produced no larvae at all. The adult flies died during the sixth week of life. Dissections at this time showed a complete lack of ovarian development.

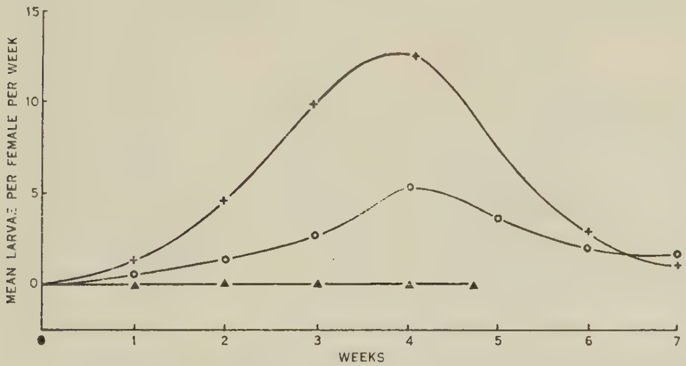

Fig. 3.—Effect of light intensity on larval production. Females of *D. tryoni* under a 150-watt electric lamp (+) produce more larvae per week than females under diffused daylight (O). No larvae were obtained from females under conditions of complete darkness (▲).

Wide variations about the weekly means in series *A* and *B* are very marked. Evidently a great deal of this variation should be considered as inherent in the species. Numerous dissections revealed a wide variation in the numbers of ovarioles per ovary, even in the same female. It is not known whether this affects the oviposition rate. Another source of variation is undoubtedly the smallness of the samples.

III. Influence of Light Intensity on Mating Activity of D. tryoni

During early work, the impression was gained that the changing light intensity prior to darkness was necessary to stimulate mating behaviour and hence necessary for a successful breeding cycle. In attempting to determine whether this was so, attention was focused on mating behaviour, which has not previously been recorded. A brief description of the mating behaviour of *D. tryoni* is therefore presented in order to make clearer the work on light intensity and mating activity to follow.

(a) *Mating Behaviour*

In the following sections it will be shown that the initial stimulus for the onset of mating activity is the falling light intensity prior to darkness.

Recordings of the time of commencement of mating activity in the laboratory at the middle of each month yielded the observations in Table 2. The time of sunset on each of the days in question has also been included for comparative purposes. These observations support the theory that the light intensity at which mating activity commences is correlated with the fading intensity of light towards the end of day.

Table 2

COMMENCEMENT OF MATING ACTIVITY

Date	Mating Activity Commenced	Sunset	Time to Sunset	Lamp Switched Off
June 15, 1949	4.45 p.m.	4.52 p.m.	6 min.	4.30 p.m.
July 31, 1949	5.00 p.m.	5.14 p.m.	14 min.	4.30 p.m.
Aug. 15, 1949	5.05 to 5.10 p.m.	5.24 p.m.	14–19 min.	4.30 p.m.
Sept. 15, 1949	5.15 p.m.	5.46 p.m.	31 min.	4.30 p.m.
Oct. 15, 1949	5.30 to 5.35 p.m.	6.08 p.m.	33–38 min.	5.00 p.m.
Nov. 15, 1949	5.55 to 6.00 p.m.	6.36 p.m.	31–36 min.	5.00 p.m.
Dec. 15, 1949	6.25 to 6.30 p.m.	7.02 p.m.	32–37 min.	5.00 p.m.

(i) *Behaviour Prior to Copulation.*—The first noticeable reaction by the insects to failing light was an increase in activity in both sexes. The males always initiated this activity by commencing to 'call' by means of rapid wing vibration. The 'calls' were clearly heard as rather high, flute-like notes emitted in series, each note varying in duration from a half to two seconds and rarely longer. No marked movement occurred in the males while 'calling,' apart from short walks towards any other fly in the immediate vicinity.

This 'calling' stimulated the females ready for copulation to approach the male. In many cases females were seen to walk from one end of the cage to the other in a straight line towards the 'calling' male. When more than one male was active in the same cage, the active females flew about until apparently 'within range' of one male, when they approached him directly.

Many females ignored the stimulus from a male even when only half an inch away. This was particularly the case when newly emerged females were caged with mature, active males.

The approach of the female to the male was followed by a very variable pattern of events. Further detailed observation will be necessary before any orderly sequence can be described. The approach ended with the pair facing each other. In many cases, the female was observed to move forwards and touch the male face to face. This was followed by copulation and the pair moved upwards to rest on the dark ceiling of the cage. In other cases the male performed a few small flights about the female. The female reacted to these by raising her forelegs away from the glass and projecting her body at right angles to it as though it were keeping the male in sight. The act of copulation then followed quickly when the male, in one of his small flights, landed on the back of the female.

Sometimes the female turned away from the male, with or without circling, and the male was then seen to move forwards and 'lick' the extended ovipositor. This in turn always appeared to stimulate the male to circle about before returning to his position at the rear. Copulation often took place then when the male moved forward. Often the position was maintained until darkness. With the male oriented directly behind the female in this manner it appears possible that copulation could occur after dark although no observations showed this to happen.

(ii) *Copulation.*—The female, if in a receptive state, turned the distal end of the abdomen upward and extruded the ovipositor. The male bent its abdomen downwards and forwards so that it projected beneath the posterior end of the ovipositor. The claspers then closed on the ovipositor from the ventral aspect and the penis was inserted into the vaginal opening near the ventral tip of the ovipositor.

The act lasted as long as 45 minutes, during which the male 'called' spasmodically if other males were 'calling' in the cage. He became quiet, however, as soon as darkness fell. The stimulus of light intensity would thus appear to play the principal part in eliciting male 'calling,' the rest of the elements in mating behaviour of the male depending on the presence of the female for their release.

Where a male attempted copulation with a female in a non-receptive state, her abdomen was bent downwards, placing the ovipositor out of reach of the claspers. Simultaneously, the female attempted to comb the male from her back with her hind legs and wings. In most cases this was successful. Copulation sometimes occurred despite this procedure when a very active male managed to grasp the ovipositor during the struggle.

Occasional observations were also made on failures to carry out successful copulation, which may have been due to aberrations in the genital armature.

In these cases the female turned the abdomen downwards, extruded the ovipositor to its full extent, and preened it with her hind legs, before turning it back upwards to the male. Six futile attempts of this nature were once recorded before the female finally broke away.

This brief description enables us to draw a preliminary pattern of events in the mating behaviour of *D. tryoni.* The failing light intensity stimulates the males to 'call,' which in turn, acts as a stimulus to the females who respond by approaching the 'calling' males. The presence of the females then elicits further responses in the male, including flying, 'circling,' and 'licking.' These excite the female. No clear sequence is shown, however, in these later elements of the chain of behaviour preceding copulation. It would appear, nevertheless, that successful copulation in *D. tryoni,* as in many other animals, "is the result of a chain of interactions between specific stimuli produced by the male and adequate responses of the female which in turn stimulate the male" (Mayr 1948).

This account of mating behaviour indicates that at least four senses are involved, the auditory, visual, gustatory, and tactile. It is suggested that the olfactory sense may also play some part in copulation after dark (see Mayr 1950).

It is of interest to note that two at least of the movements discovered by Sturtevant (Mayr 1946) to be part of the pre-copulatory chain in *Drosophila* sp., viz. 'licking' of the ovipositor and 'circling,' are also present in the mating behaviour of *D. tryoni.* Wing movements were not observed, apart from the vibrations of the 'calling' males. These appeared, however, to serve solely as auditory stimuli.

(*b*) *Light Intensity as a Stimulus to Mating Activity*

Four experiments were devised. The first three were planned to determine the effect on behaviour of various combinations of light intensities, both during normal mating and at other times. The fourth experiment aimed to obtain a quantitative estimate, in terms of larval production, of the necessity of the dusk period for a successful breeding cycle.

(i) *Elimination of Dusk Period.*—Three cages, each containing six pairs of sexually mature adults under the conditions of light, food, and other treatment described for series *A*, were subjected to continuous artificial light (150-watt lamp) during their normal mating period (5.40 to 6.15 p.m.). Observations were carried out for three consecutive evenings. Three similar cages kept under normal experimental conditions acted as controls.

Mating activity occurred each evening in the control cages but no signs of mating behaviour were observed in the cages under continuous light. When the light was turned off at 6.15 p.m. the room became dark and normal activity ceased quickly in both sets of cages. Observation by torchlight for some time afterwards showed all flies to be inactive apart from continuing copulations occurring in control cages.

(ii) *Simulation of Dusk Period at Times other than Normal.*—Attempts were made to induce mating activity at times other than sunset by simulating a change in light intensity analogous to dusk. Light was cut off, at successive minute intervals, by raising a blind from the bottom of the window. This procedure was carried out for five days.

Mating behaviour was stimulated in the three cages half an hour early (5.10 p.m.) on the first evening. Further attempts on later evenings were successful to the extent of starting mating activity at 4.50 p.m. (50 minutes early) and 4.30 p.m. (1 hour 10 minutes early).

Attempts at times earlier in the day gave negative results. It seems possible that a minimum number of hours of exposure to light during the day may be required before mating can be initiated.

(iii) *Effects of Alternating High and Low Light Intensities during the Period of Mating Activity.*—The flies in three of the cages used in the above two experiments were allowed to commence mating activity at the normal period until all were fully active. The light intensity was then varied with the following results:

Light switched on	Mating behaviour ceased
Light switched off	Mating behaviour recommenced
Light switched on	Mating behaviour ceased
Light switched off	Mating behaviour recommenced
Room darkened	Mating behaviour ceased
Room back to 'dusk'	Mating behaviour recommenced

This procedure was carried out for three consecutive evenings and yielded the same results each time. Also, when mating behaviour was initiated earlier by covering the window as outlined previously, the flies again responded as above. When the window was uncovered mating behaviour ceased but recommenced at the normal period later.

It should be noted that when the light was switched on, mating activity ceased quickly. Individuals copulating soon broke apart. When the light was switched off again, however, periods of up to two minutes were commonly noted before the males became fully active again. When the light was left on for more than a few minutes mating activity did not recommence when the room was returned to 'dusk' conditions.

(iv) *Importance of the Dusk Period in the Breeding Cycle.*—The following experiment was designed to test the possibility of copulation occurring under any other conditions than those observed and hence the relative importance of the dusk period in the breeding cycle of the insect.

The window was covered completely, except for a small aperture allowing light to pass into a covered box, into which cages could be placed in the evening without upsetting light relations in the rest of the room. Two series, each of five cages, with six pairs of flies to the cage, were then exposed to the following conditions:

Series *X*.—Seven and one-half hours of light (150-watt lamp), ½ hour of dusk in the window box, 16 hours of darkness.

Series *Y*.—Eight hours of light (150-watt lamp), 16 hours of darkness.

The flies were all of the same age and conditions of treatment remained identical with those outlined in earlier work. As in Section II(*a*), apples were placed in the cages on Monday and Thursday of each week and the larvae in each apple were counted after reaching an advanced stage in development (9 days after placing the apple in the cage).

TABLE 3

IMPORTANCE OF THE 'DUSK PERIOD' IN THE BREEDING CYCLE

	Dusk Period Included Larvae per Week						Dusk Period Excluded Larvae per Week				
Cage	1	2	3	4	5	Cage	1	2	3	4	5
X1	2	10	19	11	17	Y1	0	0	0	0	0
X2	3	12	19	13	15	Y2	0	0	0	0	0
X3	3	9	21	8	5	Y3	0	0	3	7	5
X4	15	28	37	15	10	Y4	0	0	0	0	0
X5	0	25	38	10	8	Y5	0	0	2	7	8
Mean	4.6	16.8	26.8	11.4	11		0	0	1	2.8	2.6
St. dev.	6.32	8.98	9.79	2.70	4.35						
Coeff. var. (%)	121	53.5	36.5	23.6	39.5						
Mean/ fem.	0.77	2.8	4.4	1.9	1.8		0	0	0.16	0.47	0.43
Overall mean per cage	14.12 larvae						1.28 larvae				

Week 1 in every case represents fourth week of life.

The results are shown in Table 3 and Figure 4. The differences are striking. Each cage of series *X* averaged 14.1 larvae over the period of the experiment. The corresponding figure for series *Y* was 1.3 larvae per cage.

(*c*) *Effect of Light Intensity Throughout the Day on Male Mating Activity at Dusk*

Although the impression was gained that activity throughout the day was affected by light intensity, no method of measuring this was developed. The mating activity of male *D. tryoni* at dusk, however, was found to lend itself more readily to this purpose. Estimates of activity were made during the mating period for four evenings, using two series of flies. Each series contained five replicates of six pairs of mature flies all of the same age, under the conditions of experimental procedure outlined for series *A* and *B* in Section II of this paper. Series *A* was thus exposed to a light intensity throughout the day on the average eight times (200 candles per sq. ft.) that of series *B* (25 candles per sq. ft.).

Copulatory activity was found to be the best measure for our purpose, counts being taken for 5-minute intervals throughout the period of mating activity, alternating between both series of cages. The numbers of males 'calling' at any moment in each cage were also recorded. This procedure presented some difficulty, which was partially overcome by using visible wing vibration as the criterion for counting.

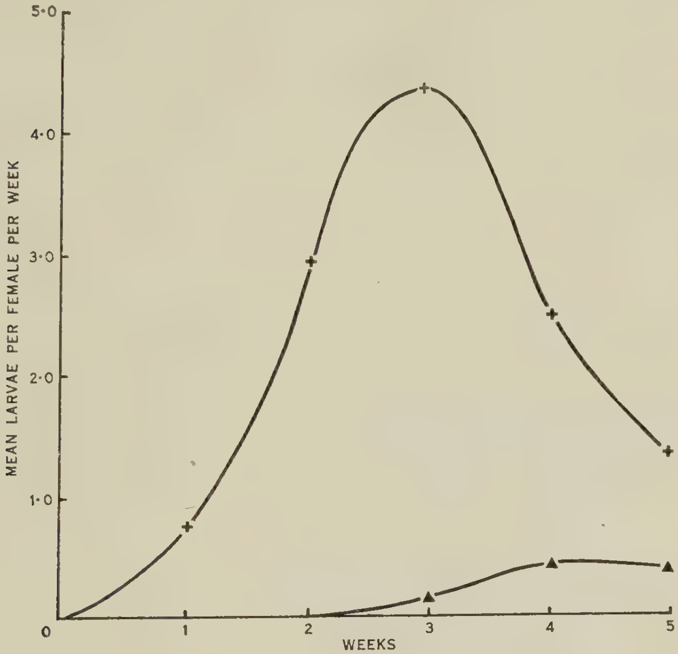

Fig. 4.—Importance of the dusk period in the breeding cycle. Females of *D. tryoni* exposed to conditions in which the changing intensity of light prior to darkness was omitted (▲) produced very few larvae, in contrast to females which were exposed to the normal pre-darkness changing light intensities (+).

Under the higher light intensity, males either copulate or attempt copulation more frequently than males that have been subjected to a lower light intensity throughout the day (Table 4). The same effect is seen in the rate of male 'calling' (Table 5).

It should be noted that Table 5 records the number of males 'calling' at the time of counting, not the number of active males in each cage throughout the evening. This does not imply that under the conditions of lower light intensity all the males in any one cage do not become active at some time during the evening.

IV. Attempts to Cross the Queensland Fruit-fly (D. tryoni) and the Solanum Fruit-fly (D. cacuminatus)

D. tryoni infests most species of the cultivated fruits of eastern Australia, while *D. cacuminatus* is only to be found in the fruits of *Solanum auriculatum* and *S. verbascifolium* (wild tobacco). Larvae of *D. cacuminatus* were collected in fruit of *S. verbascifolium* from Waterfall, N.S.W. The adults obtained from these larvae were found to breed freely in ripening tomatoes (Allman 1940) under the conditions of breeding outlined in Section II for *D. tryoni*.

Table 4

EFFECT OF LIGHT INTENSITY ON FREQUENCY OF MATING ACTIVITY

	Under 150-Watt Lamp Series *A*				Under Diffused Daylight Series *B*			
Copulations and	10	6	10	5	7	4	7	2
attempted	9	8	10	9	6	4	4	6
copulations	9	9	7	9	7	5	2	5
per 5 min.	12	9	6	12	3	5	7	4
	8	7	4	10	7	4	7	6
Mean			8.45			5.0		
St. dev.			2.04			1.68		
Coeff. var. (%)			24.1			33.6		

Significance of differences between means by '*t*' test, $P = 0.001$.

Table 5

EFFECT OF LIGHT INTENSITY ON THE NUMBERS OF MALES 'CALLING'

	Under 150-Watt Lamp					Under Diffused Daylight				
Cage	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5
Nos. of	6	5	5	5	5	2	5	2	5	4
males	6	6	4	4	4	3	3	4	3	2
'calling'	5	4	4	6	6	3	6	5	5	1
	6	4	4	6	6	4	2	3	3	2
	4	6	5	4	6	5	4	3	4	5
Mean	5.5	5.16	4.0	5.16	5.3	3.16	4.0	3.16	3.8	2.8
Grand mean			5.03					3.4		
St. dev.			1.64					1.63		
Coeff. var. (%)			32.3					47.9		

Significance of difference between grand means, using '*t*' test, $P < 0.001$.

Observations on the mating behaviour of *D. cacuminatus* showed this to be very similar to that of *D. tryoni*. The solanum fruit-fly was found to commence mating activity 10-15 minutes earlier in the evening and always appeared to be a much more active insect than the relatively sluggish *D. tryoni*. Field observations also demonstrated that the two species were sympatric. It thus

seemed of interest to carry out some crossing experiments in order to obtain some ideas on the isolating mechanisms existing to keep the species separated in nature.

Five experiments were devised in an attempt to find out whether barriers to crossing existed and the nature of those barriers. While this work was not of a comprehensive nature, suggestions for future quantitative studies are presented.

All flies used were of laboratory-bred stock and were exposed to conditions of light as described for series *A*. The main features of each experiment and its outcome are presented in Table 6.

TABLE 6

SUMMARY OF CROSSING EXPERIMENTS BETWEEN *D. TRYONI* AND *D. CACUMINATUS*

Experiment	Number of Replicates	Adults per Cage			Results
1	6	1 *D. cacuminatus* ♀	×	1 *D. cacuminatus* ♂ 1 *D. tryoni* ♂	2 copulations, conspecific
2	6	1 *D. tryoni* ♀	×	1 *D. cacuminatus* ♂ 1 *D. tryoni* ♂	3 copulations, conspecific
3	3	1 *D. cacuminatus* ♂	×	1 *D. cacuminatus* ♀ 1 *D. tryoni* ♀	2 copulations, conspecific
4	3	1 *D. tryoni* ♂	×	1 *D. cacuminatus* ♀ 1 *D. tryoni* ♀	1 copulation, conspecific
5	2	5 *D. tryoni* ♀ ♀	×	5 *D. cacuminatus* ♂ ♂	Interspecific copulations occurred; 88 eggs collected failed to hatch
	2	5 *D. tryoni* ♂ ♂	×	5 *D. cacuminatus* ♀ ♀	No copulations; 70 eggs collected failed to hatch

The only interspecific copulations occurred where females of *D. tryoni* were caged with males of *D. cacuminatus*. No fertile eggs resulted from this union, however.

Observations on the behaviour of the flies showed fairly clearly that the females of *D. cacuminatus* were attracted to the 'calling' male of the opposite species only in the absence of their own males. When given the choice of mating with a male of both species, the initial approach was always towards the male of their own species. In this respect it might be noted that the 'call' of the *D. cacuminatus* males seemed to be audibly higher pitched than that of *D. tryoni,* though this difference was not measured. Females of *D. tryoni,* on the other hand, usually moved towards the closest 'calling' male. In both

species, however, the females discontinued mating activity when the approach brought them into close contact with a foreign male.

Also noticeable was the indiscriminate attempts of males to mate with females of either species. In all observations made during this work the impression was gained that males of both species attempted copulation with anything moving in the vicinity, even each other.

In experiment 5, in the cages containing *D. tryoni* females and *D. cacuminatus* males, the females commenced approaching the males 16 days after emergence. Many copulations were attempted but these were actively resisted by the females. At 22 days, the first successful copulation was observed. The female, after initial resistance, became quiet and the act ensued, apparently normally. Numerous other copulations were noted at later dates. A sample of 88 eggs was taken from the apples. None of these hatched and no signs of development could be observed under the microscope. At 56 days, all the females were dissected and the spermathecae examined in saline. These all showed the presence of numerous, active sperm.

In cages containing females of *D. cacuminatus*, the females commenced mating activity 17 days after emergence. The males of *D. tryoni* attempted copulation repeatedly, but no successful copulations were observed up to 56 days. All the males were dislodged very quickly by the active females. Seventy eggs collected from tomatoes failed to hatch and showed no signs of development. Dissections of all the females showed the spermathecae and their ducts to be empty. Dissections of the testes of the male *D. tryoni* showed active sperm present in all cases.

It would thus appear that the barriers to crossing between the two species are complete. There appears to be some degree of discrimination in mating pairs with respect to the mating partner. This effective isolating mechanism, aided by habitat segregation to a large degree, has been reinforced by physiological differences.

Patterson (1946) showed in certain interspecific matings of *Drosophila* spp. that the vagina swells after copulation and the sperm die. Since the sperm present in the spermathecae of the *D. tryoni* females were very active, there was no apparent incapacity of the sperm in this case. A cytological study would be required to determine whether the sperm are incapacitated before meeting the egg or whether the zygote dies at a very early stage. Introductory to this, it has been found (Crawford 1949, unpublished data) that marked differences in the chromosome complement of each species do occur.

V. Discussion

Very little work appears to have been carried out on the influence of light on fecundity. Menusan (1935) working with bean weevils, showed that constant white light reduced the number of eggs deposited and that the reduction was proportional to the light intensity used. Other insects, including *Drosophila*

and the Levant house-fly, *Musca domestica vicina* Macq. (Feldman-Muhsam 1934) oviposit as many eggs in complete darkness as in normal conditions of light and dark.

An analysis of how light intensity affects fecundity in S. *tryoni* has not yet been attempted but certain interesting suggestions may point towards an explanation of the phenomenon.

In the two sets of cages, *A* and *B*, flies under the lower light intensity appeared much less active than those under the 150-watt lamp. This activity difference was difficult to express in numbers but was noticeable in the frequency of flight, of walking, and of feeding.

Flies that fed frequently appeared to regurgitate and defaecate more often than those feeding less often. This was clear from the state of cleanliness of the three sets of cages, which varied from the almost clean cages of flies under conditions of complete darkness to the constantly dirty cages of the flies under the 150-watt lamp.

Bucher, Cameron, and Wilkes (1948) reported that *Musca domestica* adults feed very little in complete darkness and maintained that chance contact with the food dishes (in the more heavily populated cages) probably explained the small percentage that did consume food in the absence of light. Baranov (1940) showed that rate of oviposition in the olive-fly, *Dacus oleae*, depended on the amount of food to which it had access. It seems possible therefore that the effects of different light intensities on fecundity in *D. tryoni* may be due to parallel differences in feeding and other activities.

The possibility that light influenced hormone production should also be considered. In insects, it would appear that the ovaries are far more under the control of endocrine action than the male gonads (Scharrer 1948). It is worth noting that in *D. tryoni*, although no female under conditions of complete darkness developed eggs, every male dissected, though in a similar shrunken, starved condition, possessed active sperm in its gonads. Light (intensity) acting through the eyes on the sinus gland in Crustacea is known to cause hormone activity affecting pigment migration within the eyes and over the body (Scheer 1948).

An explanation of the effect noted, where light intensity undergone throughout the day affected the degree of male mating activity at dusk, may also be found to be connected with the accumulation of a hormone in the blood, developed within the *corpus allatum* or some other endocrine organ directly under the control of daily light intensity.

In the field, light intensity probably never acts alone as a limiting factor in the breeding cycle of *D. tryoni*, although it may slow down egg development over a period of dull weather. For laboratory work, however, it must be conceded a place of importance in any technique of breeding, with respect to both the daily intensity and the failing intensity at the end of the day.

VI. Acknowledgments

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WOUND HEALING IN THE GUT OF THE COCKROACH *PERIPLANETA*

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Summary

Incisions in the fore-, mid-, or hindgut of the cockroach *Periplaneta* heal in a large percentage of operations. The processes of healing are described. They involve firstly the formation of wound tissue from haemocytes, and later the participation of the epithelium. Operations performed on nymphs do not appear to hinder moulting, but the wounds are usually still conspicuous in the resulting adults, except in operations on the midgut, in which healing may be complete. Comparisons are made between the processes of wound healing, neoplasms, and the normal changes of the epithelium at moulting.

I. Introduction

The opinion is current among entomologists that operations involving punctures of the alimentary tract of insects are usually fatal. Thus, Beard (1945, p. 516) reports that larvae of the Japanese beetle "cannot tolerate any injury to the gut which permits the escape of gut bacteria into the blood." Of the same insect he writes (1949, p. 86) "fatal infection invariably occurs if the gut is punctured." However, Storey (1933) carried out experiments with the jassid *Cicadulina mbila* designed to puncture the midgut with a sterile needle, and a fair percentage of the treated insects did not succumb to the effects of the operation.

There seemed to be two possible explanations for Storey's report: (*a*) that the gut of the jassids is relatively sterile (cf. Duncan 1926) and the operated insects did not therefore suffer from septicaemia, or (*b*) that the operation is not, in fact, a fatal one in some insects, if performed with care.

It was decided to test the second of these suggestions, using an insect large enough for operative techniques to be carefully carried out, and one in which the gut was well populated with microorganisms. The cockroach *Periplaneta americana* (L.) meets these requirements and has been used exclusively in this work.

It was found that this cockroach survives quite extensive injuries to the crop, caeca, midgut, and hindgut, and that the wounds heal in a large percentage of cases. No record could be found in the literature of the healing of wounds of the alimentary tract of insects, and so it has seemed worth while to report the following details on the mechanism of regeneration, even though the analysis is based only on histological and histochemical observations.

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II. Methods

Thriving colonies of the cockroach were maintained in the laboratory at approximately 27°C. The insects were lightly infected with nematodes and ciliates but these did not affect the present observations. Adults were anaesthetized with carbon dioxide and immediately operated upon with sharpened No. 12 hard steel needles. Recovery from the anaesthetic occurred within 5 minutes and the insects were subsequently kept in 2-pint glass jars and supplied with food and water. Aftei various periods from a few hours to more than 8 months the operated tissues were examined, either as tissue spreads following staining in acetic orcein or methylene blue, or most frequently after fixation in alcoholic Bouin's fluid. Zenker's fixative was used with methyl green-pyronin stained preparations. Paraffin sections cut at 10 μ were generally stained in Mallory's triple connective stain or by Bodian's protargol method. Tracheal injections were prepared by the method of Hagmann (1940). Attempts to watch the healing process by removing part of the cuticle and replacing it by a small piece of plastic cover slip were useful, but did not add to the conclusions drawn from other methods.

III. Observations

(*a*) *General*

The survival rate until fixation for study for a total of 102 gut operations was 84 per cent. These were divided among the regions of the gut as follows: foregut, 86 per cent. survival; midgut, 77 per cent.; hindgut, 87 per cent. In a series of much more extensive wounds (wounds greater than 1 mm. in length) the survival rate dropped to 30 per cent. The greatest post-operative mortality occurred within 48 hours.

The principal features of regeneration either of large or small wounds are similar in all regions of the gut examined. Differences will be mentioned following a general description.

Within a few hours after the operation (the shortest time was not determined) haemocytes begin to accumulate at the wound. This accumulation leads to the formation of "wound tissue" (Plate 1, Figs. 3 and 4) and continues until the injury is plugged and until any particular matter that has escaped into the haemocoele is completely surrounded. The haemocytes involved appear, on the basis of nuclear size, to include the three cell types described by Ermin (1939), but as they undergo striking changes in shape when they become incorporated into the wound tissue, it is difficult to be certain of this point. The haemocytes may become flattened, elongate, or spheroidal, depending upon their position.

Blood cells, containing phagocytosed particles of Chinese ink, which had been injected into the haemocoele 24 hours previously, took part in the formation of wound tissue (Plate 1, Fig. 4). But neither Chinese ink nor a variety of vital dyes were taken up by the wound tissue once it was formed.

The muscles of the wounded organ generally contract around the site of the injury and their nuclei may become pycnotic. This contraction may cause the connective tissue to become more conspicuous. The epithelium of the organ in the vicinity of the injury becomes vacuolate and the cytoplasm of damaged cells becomes necrotic. Generally, however, the surrounding epithelial cells do not show signs of injury, nor do they take part in the initial plugging of the wound in these early stages. Mitoses have been observed only rarely in the wound tissue and did not seem to be increased by colchicine. Rough estimates have not shown any marked decrease in the numbers of circulating haemocytes even though tremendous numbers may take part in the formation of the wound tissue. This is less surprising when it is recalled that Beard (1949) found no significant decrease in numbers of blood cells per cu. mm. in larvae of Japanese beetles in which extensive haemorrhage had been induced, in comparison with normal larvae. Nor was there a marked increase in the present experiments in mitotic activity of circulating haemocytes either 5 hours or 24 hours after the operation. Probably a number were mobilized from a previously sedentary state, and replacement took place slowly by mitosis in the haemolymph.

In this first stage of healing, then, the injured epithelium may undergo some destruction, but all of the constructive changes result from the activity of the haemocytes. This situation continues for about 3 days. At the end of this time, all necrotic tissue and cellular debris is encapsulated by haemocytes, and tissues and even normal organs, such as malpighian tubules, muscles, tracheae, and nerves may be incorporated in the wound tissue. Encapsulation is particularly marked where nematode parasites penetrate the haemocoele through a hindgut incision.

By the third day activity is evident on the part of the epithelium and this continues slowly until the wound is healed. This may require a period of months or in adults may even never occur. During this time the wound is undergoing contraction, as indicated by the pattern of tensions in the surrounding tissue (cf. Weiss 1949, p. 175). The epithelial cells of the fore- and hindgut surrounding the wound have occasionally been observed in mitosis. Amitosis has not been observed either in the epithelium or in the wound tissue. Granules staining with pyronin appear in the cytoplasm of the epithelial cells within 3 days. They are still found there in very reduced numbers after 6 weeks. It is possible that these granules may be concerned with the regenerative process as suggested by Peters (1945) for regenerating wounds in rat skin following burning. In the midgut, replacement clearly takes place from the regenerative nidi, the cells of which can be seen in mitosis more frequently in the region surrounding the injury than in the other parts of the organ. The new epithelial cells slowly migrate across the wound, and so separate from the wound tissue an eschar (Plate 1, Fig. 2) consisting of necrotic tissues.

This wound tissue may be of considerable bulk, but tracheae do not penetrate it unless they were present before the tissue was formed. Nor is it innervated. In wounds of the crop the *nervus recurrens posterior* has been

seen to run through the wound tissue without impairing its ability to stain with methylene blue. The wound tissue attains its maximum size in 3 or 4 days and then, after about 3 weeks, begins to decrease. When epithelial continuity has become completely re-established the wound tissue is reduced, and it is surmised that the haemocytes become circulating cells again. Thus, in an old wound, the wound tissue may be represented by a thin epithelium-like layer (cf. Plate 1, Fig. 1). There is no information of the biochemical or metabolic change in the insect following wounding. One observation, however, may give a lead to the study of these problems. The epithelial cells of the hindgut of the normal adult *Periplaneta* contain a number of granules, which Waterhouse (unpublished observations) has demonstrated to consist of calcium and other metal salts (largely phosphates). These appear unchanged up to 3 days following an operation. Then they often disappear in some of the hindgut epithelial cells surrounding the wound. They may not be replenished even 8 weeks later.

Some operations have been performed on last-instar nymphs. Moulting occurred normally from a few days to 2 months after the operation. In wounds of the crop or hindgut wound tissue was evident even after the moult. In some wounds of the midgut, however, regeneration was complete and no sign of the wound could be detected in the resulting adult. This difference might be expected to result from the greater regenerative power of the midgut epithelium.

The healing process in each of the regions, fore-, mid-, and hindgut, shows slight variations from the general description given above. These will now be described.

(*b*) *Foregut*

The crop is the most suitable organ for the study of the details of regeneration, particularly because of the simplicity of its tissues. These are thin enough for whole mounts to be used to show very clearly the contraction patterns as the wound heals. Presumably owing to the arrangement of the muscles, the eschar, consisting of necrotic cells originating both from the injured epithelium and the inner layers of the wound tissue, is forced into the lumen of the crop where it remains at least for several months. The regenerating epithelium must force its way between this eschar and the wound tissue which projects into the haemocoele. Evidence for such migration can be seen in Plate 1, Figure 2. Soon after the wound tissue is formed a non-cellular, basophilic layer appears between the wound tissue and the scab. It is continuous with the chitinous intima and stains in the same way. It is not possible to say whether it is, in fact, chitinous, nor whether it is produced from the wound tissue, which is of mesodermal origin, or from the epidermal cells that have migrated between the wound tissue and the necrotic tissues. It should be noted that a similar layer is produced in the hindgut, but it is quite absent from a healing wound in the midgut.

In a number of wounds of the crop the organ was found to be distended and to contain a large air bubble for some time after the operation. This may have assisted in the healing process by counteracting the tendency of the muscles to contract; in vertebrate gut wounds attempts are usually made to keep the organ deflated (Whipple 1943).

(*c*) *Midgut*

The regenerative processes of the caeca and of the midgut are essentially similar and will be described together. The eschar is never formed in the lumen as it is in the crop, but is always forced out into the body cavity. However, epithelial breakdown is much more extensive in the midgut and cellular debris in quantity is forced into the lumen where it occupies the extra-peritrophic space and undergoes histolysis. The regenerative nidi surrounding the wound supply the cells for the regenerating epithelium (Plate 1, Fig. 3). As they move over the surface of the wound tissue to replenish the epithelium they rapidly gain a striated border characteristic of the normal epithelium; when they occur in the anterior part of the midgut they contain typical argentophil inclusions, but these are fewer in number than in normal cells.

(*d*) *Hindgut*

As pointed out above, the necrotic tissue (eschar) is forced into the lumen of the crop, but into the haemocoele if the wound is in the midgut. The eschar may be either in the lumen or in the haemocoele in hindgut wounds, but the latter is more usual. This difference is presumably due to the forces of muscular contraction and of the pressure of the hindgut contents. The wound tissue is noticeably more marked in hindgut wounds than in either crop or midgut wounds, mainly owing to the abundance of microorganisms that escape into the haemocoele. Wound tissue frequently includes malpighian tubules, fat body, and other tissues. Usually such included organs have a normal appearance but some fat body seems to undergo lysis of cells except for the bacteroid-containing cells, which may then occur as isolated groups surrounded by wound tissue.

The most noteworthy feature of the hindgut epithelium during regeneration is the depletion of granules, thus leaving large vacuoles in the epithelial cells.

IV. Reactions of Wound Tissue

(*a*) *Transplantation*

It will be apparent that the wound tissue described above has some of the histological characteristics of neoplastic growth. An attempt was therefore made to determine whether wound tissue had the principle feature of neoplasms, namely the ability to continue to grow when transplanted. Wound tissue from fore-, mid-, and hindgut wounds was therefore transplanted under the abdominal tergites of other adult *Periplaneta*. Controls were of two types.

Either small segments of normal gut tissue (caecal fragments proved to be the most useful) or small pieces of paraffin wax were implanted in the same way into other hosts. In all cases the reaction was the same. The foreign tissue (or wax) was encapsulated by host haemocytes (Plate 1, Fig. 5) but did not proliferate. It may thus be concluded that the histological similarity between wound and neoplastic tissue is not indicative of any more basic similarity.

(*b*) *Effect of Haemocytic Anticoagulants*

In view of the importance of haemocytes in the wound healing process, an attempt was made to determine the effect of inhibiting coagulation of these cells. Clotting in *P. americana* is said to be of the first type of Yeager and Knight (1933), that is, only the cells clot without the intervention of plasma. Beard (1950) has shown that ascorbic acid, in concentrations of about 0.01M, inhibits coagulation in the species he examined. This substance has an inhibiting effect on coagulation in *P. americana.* Injection into the haemocoele of 0.1 ml. of 0.01M ascorbic acid resulted in a fairly high mortality. But sufficient survived for operations to be performed on a number of treated adults 4 and 24 hours following the injection. Mortality resulting from these operations was more than twice normal. Only one individual survived of six in which the operation was performed 4 hours after injection. In this, and in individuals into which paraffin wax beads were implanted following injection of ascorbic acid, the accumulation of haemocytes was very markedly reduced, and practically no wound tissue was formed (Plate 1, Fig. 6). The wounds were not plugged and this undoubtedly was the cause of the high mortality. These observations lend support to the hypothesis that the haemocytes play the dominant role in the first stage of the healing of wounds of the alimentary tract.

V. Discussion

The processes of regeneration following gut wounds are essentially similar to those described following epithelial wounds, which Ermin (1939) described in the same species, and which Wigglesworth (1937) described for *Rhodnius.* Moreover, the reaction of the haemocytes to form wound tissue is similar to that produced by the introduction of any foreign body into the haemocoele. The histological similarity between the wound tissue and neoplasms has been mentioned by Scharrer and Lochhead (1950). In wound tissue there is apparently no de-differentiation of cells, which is presumably a characteristic of true neoplasms, and in recent cytological studies of *Drosophila* tumorous growths the same has been found to be true. Thus Hartung (1950) has concluded that "little or no evidence of mitotic activity is apparent and the growth in all cases appears as discrete nodular clumps rather than as invasive proliferations." It seems quite probable that blood cells of many insects have the property of spontaneous aggregation to form discrete bodies, and that many of the bodies described as "blood-forming organs" (Cuénot 1898), phagocytic

organs (Cuénot 1895), or the body of unknown function illustrated in *Macropanesthia* (Day 1950, Plate 4, Fig. 24) are nothing but aggregations of haemocytes. A further comparison between wound healing and the behaviour of the epithelium at moulting is noteworthy; the main difference is that in wound healing the processes are localized instead of occurring generally over the whole epithelium, but as Wigglesworth (1937) also indicated, the processes have many properties in common.

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Explanation of Plate 1

Plate 1

Photomicrographs of 10-μ sections of healing gut wounds in *Periplaneta* adults taken on 35 mm. film using 8x ocular and 33x objective.

Fig. 1.—Wound tissue surrounding a paraffin pellet implanted into abdominal haemocoele for 8 weeks. Note that the wound tissue is reduced compared with that in Figure 5, and that it has formed a pseudo-epithelium and a layer of necrotic tissue (on the right) against the pellet. Bodian preparation.

Fig. 2.—Wound tissue of crop in which incision had been made 2 weeks previously. The epithelial layer has penetrated between the wound tissue and the eschar (at top left) and has produced a clear, chitinous (?) layer against the eschar. Tissue at the bottom right is fat body. Bodian preparation.

WOUND HEALING IN *PERIPLANETA* GUT

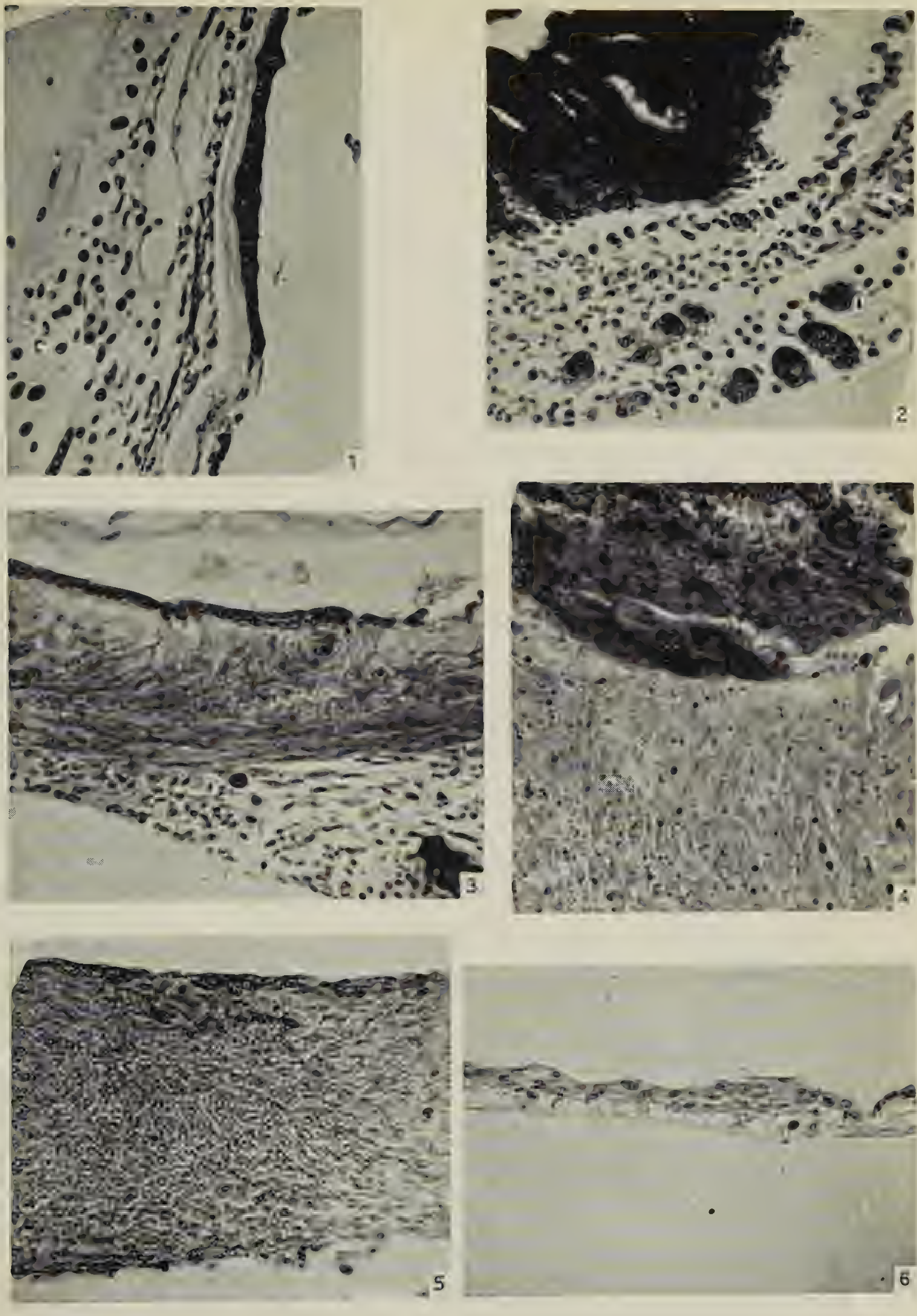

Fig. 3.—Midgut wounded 2 weeks previously. Note that the regenerative cells have almost completely replenished the midgut epithelium, but that the wound tissue is still well developed. Near the bottom right is some tissue debris encapsulated by the wound tissue. Bodian preparation.

Fig. 4.—Wound tissue formed over crop wound of insect into which Chinese ink had been injected 24 hours previously. Note that the phagocytic haemocytes have taken part in the formation of both the wound tissue and the eschar. Mallory's stain.

Fig. 5.—Encapsulation by haemocytes of paraffin pellet (at the top but now dissolved) implanted in haemocoele for 24 hours. Note the bulk of the wound tissue compared with that in Figures 1 and 6. Note also the variations in the cells depending upon their position in the wound tissue.

Fig. 6.—Encapsulation by haemocytes of paraffin pellet (dissolved during preparation of section) implanted for 24 hours in insect into which ascorbic acid had been injected 4 hours before implantation. Note the sparseness of the wound tissue in comparison with that in Figure 5.

THE PRECIPITATION OF GELATIN BY ETHANOL, AND ITS USE IN THE ESTIMATION OF PROTEOLYTIC ACTIVITY

By W. G. Crewther*

[*Manuscript received October* 25, 1951]

Summary

Within certain limits of gelatin concentration, pH, temperature, salt concentration, and ethanol concentration, the percentage light transmitted through precipitated gelatin solutions is an exponential function of the gelatin concentration. It is concluded that the mean micelle size of the precipitated gelatin is independent of the gelatin concentration within these prescribed limits.

Two methods for estimating proteolytic activity have been based on the precipitation of gelatin by ethanol. In the first method buffered solutions of gelatin, digested by the protease under standard conditions, are precipitated by an ethanol-buffer mixture, the decrease in turbidity as the result of proteolysis providing a measure of its activity. The relationship between the logarithm of the percentage transmitted light and the enzyme concentration approximated to a straight line over a considerable range of enzyme concentrations. The second method depends on the gravimetric determination of the amount of isoelectric gelatin rendered soluble in 80 per cent. ethanol by the action of the enzyme. Using solutions of protease from the mould *Aspergillus oryzae*, this value was found to be proportional to the amount of enzyme present if dilute solutions of enzyme were employed. The relationship between the amount of gelatin made soluble and the enzyme concentration varied according to the type of protease used.

These methods have been compared with other conventional methods for estimating proteolytic activity and for a number of proteases have been found to provide equal or greater sensitivity.

I. Introduction

Most samples of gelatin are polydisperse, owing to degradation in the course of their preparation. Thus by fractional precipitation Mosemann and Ligner (1944) obtained preparations having mean molecular weights of 16,000 and 89,000 respectively. It has also been shown by Briefer (1929) that the isoelectric point of gelatin depends on the manner of preparation. However, Jirgensons (1942) found by precipitation titration of degraded gelatin that the molecular weights determined in this way correspond approximately with those determined by cryoscopic methods, while fractionation of gelatin gives products having the same refractive index and isoelectric point (Straup 1931). It would appear, therefore, that the amino acid composition of the small gelatin molecules corresponds fairly closely with that of the larger molecules, although these molecules cover a wide range of molecular weights.

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If, as the data quoted above indicate, the complexity of gelatin is due mainly to variation in molecular weight, rather than to variation in amino acid composition and arrangement, information concerning the precipitation of gelatin with ethanol might provide a basis for following the digestion of gelatin by proteolytic enzymes.

A study has therefore been made of the relationship between gelatin concentration and light transmission under different conditions of pH, salt concentration, temperature, and concentration of the precipitant. Using these results, an optical method has been developed for determining protease activity.

For experiments requiring greater accuracy and reproducibility, it has been found advantageous to estimate the amount of gelatin precipitated gravimetrically, the precipitation with ethanol being carried out at the pH of minimum solubility of the gelatin. The sensitivity of this method has been compared with that of the turbidimetric method and other established methods for estimating protease activity.

II. Methods

(*a*) *Preparation of Gelatin Solutions and Method of Precipitation*

For these experiments a bulk lot of gelatin* was used. Buffered solutions were prepared by boiling a solution of succinic or acetic acid that had previously been partly neutralized with NaOH, and adding gelatin after removal from the burner. On cooling, the pH was adjusted with NaOH, using a glass electrode, the solution was made up to volume, and the pH checked. Dilute solutions were prepared by dilution with buffer of the same concentration and pH.

As it was hoped to use the information obtained for developing methods of protease estimation, rapid precipitation of the gelatin was employed. The precipitant was blown into the gelatin solution from a fast-flowing pipette, the jet of liquid striking the tube just above the surface of the solution, thereby causing rapid mixing.

(*b*) *Turbidimetric Experiments*

Changes in the turbidity of gelatin solutions were followed in an Evelyn type photometer using a green filter, or in a Coleman spectrophotometer at a wavelength of 535 mμ. Precipitation of the buffered gelatin solutions was carried out in 25 by 200 mm. test tubes standing in a constant-temperature water-bath, the precipitant mixtures being maintained at the same temperature as the gelatin. Unless otherwise stated, readings were taken 1 minute after precipitation in order to allow air bubbles to rise to the surface.

(*c*) *Gravimetric Experiments*

An Oertling air-damped balance was used for weighing the centrifuge tubes used in these experiments. The order of accuracy was $\pm$ 0.2 mg. Details of procedure are provided in the experimental section.

* Supplied by Davis Gelatine Pty. Ltd., Sydney.

III. Experimental and Results

(a) The Precipitation of Gelatin

Aliquots (2 ml.) of solutions having differing gelatin concentrations were precipitated by 10 ml. of ethanol at 40°C. and the light transmission measured after 1 minute. The gelatin was at a pH of approximately 5.0 and contained no added buffer. From Figure 1 it is apparent that a straight-line relationship exists between the gelatin concentration and the logarithm of the percentage light transmitted up to concentrations of about 0.4 per cent. gelatin, at which point the slope of the curve changes sharply. As would be expected, the gelatin concentration required for the appearance of measurable turbidity increased with decreasing concentrations of ethanol in the final mixtures.

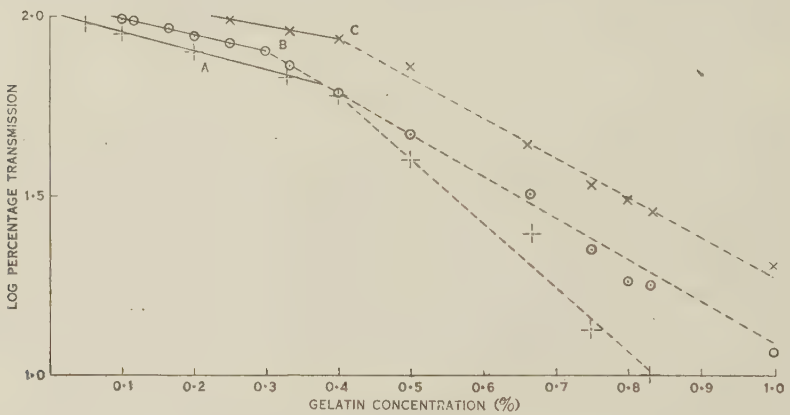

Fig. 1.—Relationship between percentage transmission and gelatin concentration.
A, 2 ml. gelatin solution + 10 ml. ethanol.
B, 5 ml. gelatin solution + 10 ml. ethanol.
C, 6 ml. gelatin solution + 10 ml. ethanol.

The transmission values of precipitated gelatin suspensions were found to change with time (Fig. 2) and, in general at low gelatin concentrations, the transmission values decreased with time whereas at high gelatin concentrations there was a steady increase in transmission. At intermediate concentrations the transmission has been found to decrease to a minimum, then increase steadily.

The effect of pH on the precipitation was followed by precipitating 0.3 and 0.75 per cent. gelatin solutions in 0.1M succinate buffer, with five volumes of ethanol, over a range of pH values. The relationship between pH and log percentage transmission is shown in Figure 3. In each case an atypical pH-precipitation curve was obtained. With 0.3 per cent. gelatin a single minimum value was obtained indicating maximum precipitation at pH 4.9, whereas with 0.75 per cent. gelatin two minima were obtained, at pH 4.3 and 7.5 respectively.

Probably this is related to the fact that at pH values near the isoelectric point a non-linear relationship exists between gelatin concentration and log percentage transmission for high gelatin concentrations.

The range of gelatin concentrations giving a rectilinear relationship between log percentage transmission and gelatin concentration varies considerably with pH, the most limited range being at the pH of minimum solubility. Using a different batch of gelatin, it has been shown that the limiting gelatin concentrations giving this straight-line relationship at pH values of 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, and 8.0 were 1.2, 0.8, 0.5, 0.4, 0.8, 1.0, and 1.2 per cent. respectively. The turbidity produced by a particular sample of gelatin under standard conditions of concentration, temperature, pH, etc. will of course depend on the extent of degradation of the gelatin.

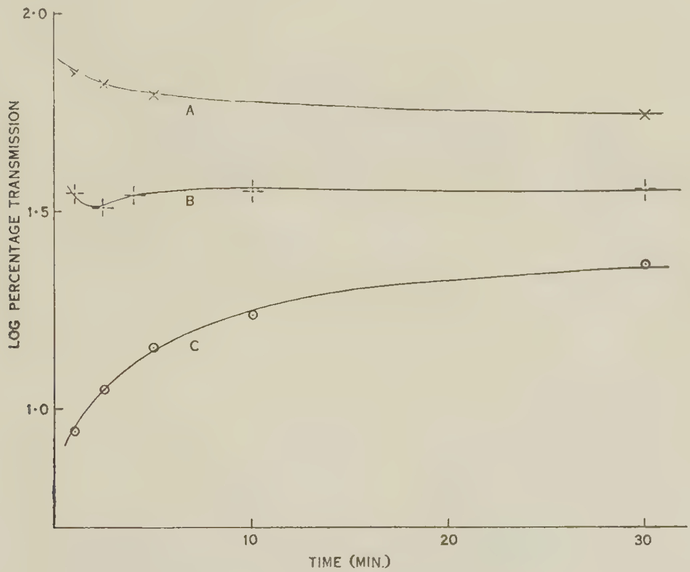

Fig. 2.—Variation in log percentage transmission with time. *A*, 2 ml. 0.2 per cent. gelatin + 10 ml. 80 per cent. ethanol. *B*, 2 ml. 0.5 per cent. gelatin + 10 ml. 80 per cent. ethanol. *C*, 2 ml. 1.0 per cent. gelatin + 10 ml. 80 per cent. ethanol.

Gelatin is known to retain some of its gel structure in solution at temperatures less than about 35°C. The precipitation of a dilution series of gelatin solutions in 0.1M succinate buffer at pH 7.0 was therefore carried out at a number of temperatures, both the gelatin solution and the precipitant being maintained at the experimental temperature. From Figure 4 it is apparent that at 30°C. the range of gelatin values giving a straight-line relationship between concentration and log percentage transmission was less than at 40°C.

The possible influence of uneven local ethanol concentrations was also investigated by precipitating aliquots (2 ml.) of the gelatin solutions with mixtures (10 ml.) of ethanol and 0.1M succinate buffer at pH 7.0, which

contained 80 and 90 per cent. ethanol to give final ethanol concentrations of 66.7 and 75.0 per cent. respectively. Aliquots (5 ml.) of the gelatin solutions

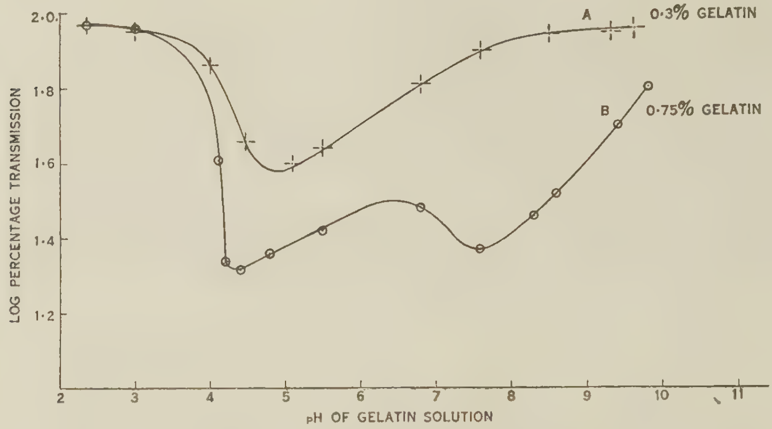

Fig. 3.—Variation in log percentage transmission with pH.
A, 2 ml. aliquots of 0.3 per cent. gelatin precipitated with 10 ml. ethanol.
B, 2 ml. aliquots of 0.75 per cent. gelatin precipitated with 10 ml. ethanol.

were also precipitated with 10 and 15 ml. of undiluted ethanol, to give the same final ethanol concentrations. Since the precipitation of 2 ml. of 2 per cent.

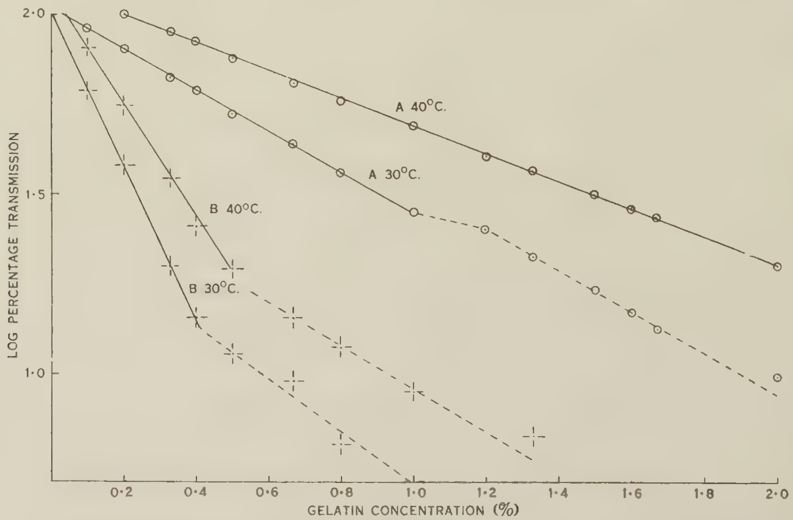

Fig. 4.—Effect of temperature on the precipitation of gelatin with ethanol.
A, 2 ml. gelatin solution + 10 ml. 80 per cent. ethanol at 40 and 30°C.
B, 5 ml. gelatin solution + 15 ml. ethanol at 40 and 30°C.

gelatin by 10 ml. of 80 per cent. ethanol is equivalent to precipitation of 5 ml. of 1 per cent. gelatin by 10 ml. of ethanol, it might be expected that the

slope of curve *C* in Figure 5 would be twice that of curve *D*. The ratio of the slopes of curves *C* and *D* is in fact 1.8. Similarly, instead of the ratio of 1.5, which would be expected for the slopes of curves *A* and *B*, the experimental value is 1.35. There is no evidence that the use of buffer-ethanol mixtures improved the approximation to a rectilinear relationship between gelatin concentration and log percentage transmission, nor was the range of final gelatin concentrations giving this relationship increased. It does, however, permit variation of the working range of transmission values for a particular initial gelatin concentration.

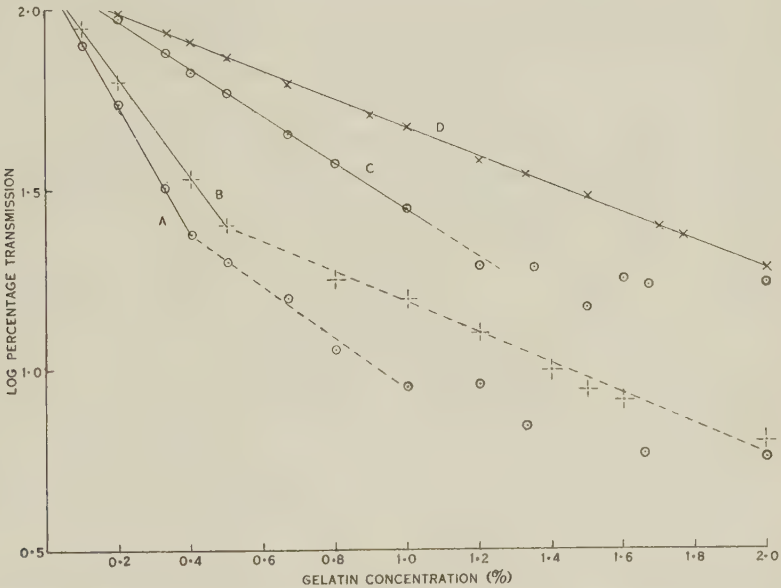

Fig. 5.—Effect of dilution of the precipitant with buffer on the concentration range of the straight-line relationship between log percentage transmission and concentration of gelatin before precipitation.
A, 5 ml. gelatin solution + 15 ml. ethanol.
B, 2 ml. gelatin solution + 10 ml. 90 per cent. ethanol.
C, 5 ml. gelatin solution + 10 ml. ethanol.
D, 2 ml. gelatin solution + 10 ml. 80 per cent. ethanol.

(*b*) *Estimation of Protease Activity Turbidimetrically*

The concentration of gelatin in the buffer substrate for this estimation will depend on the sample of gelatin used, the sensitivity of the photometer used, and the pH of precipitation. In general the gelatin concentration was such that on precipitation under the standard conditions the percentage transmission was between 10 and 20.

(i) *Reagents.*—The buffer substrate consisted of 2 per cent. gelatin (or any other suitable concentration) prepared in 0.1M succinate buffer at pH 7.0 without boiling the gelatin. The precipitant contained four volumes of ethanol

mixed with one volume of 0.1M succinate buffer at pH 7.0. As crystals of succinate appear after standing for long periods at room temperature, the precipitant is prepared immediately before use.

(ii) *Procedure.*—Aliquots (2.0 ml.) of buffer substrate in 25 by 200 mm. test tubes and a stoppered flask of precipitant were allowed to equilibrate in a water-bath at 40°C. The enzyme solution (0.2 ml.) was added to the substrate, which was shaken and incubated for 1 hour at 40°C. Precipitant (10 ml.) was then blown into the tube, the contents of which were transferred to a cuvette and the light transmission measured 1 minute after precipitation. The cuvette was rinsed with water and 80 per cent. ethanol after use, and drained on absorbent paper. When assaying a series of enzyme solutions it is convenient to use several cuvettes matched with the control cuvette. A single blank tube containing succinate buffer (2.2 ml.) and precipitant (10 ml.) may be used where very dilute purified enzyme solutions are being used. Otherwise a separate blank containing succinate buffer (2.0 ml.), enzyme (0.2 ml.), and precipitant (10 ml.) is required for each test.

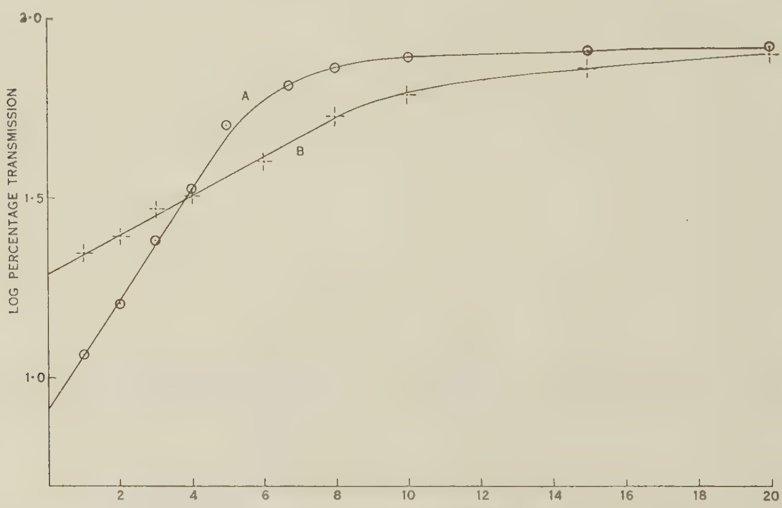

Fig. 6.—The turbidimetric estimation of protease activity. Relation between percentage transmission and concentration of mould protease.
A, 30 seconds after precipitation.
B, 30 minutes after precipitation.

The activity of the protease solution was obtained by comparison of the transmission value with a standard dilution curve for the particular protease being tested. In Figure 6 log percentage transmission is plotted against concentration of protease from *A. oryzae* expressed as arbitrary units. Graph *A* was obtained using a period of 30 seconds between precipitation and reading the turbidity, whereas for graph *B*, a 30-minute period was used. In each case a straight-line relationship was obtained over a considerable range of enzyme

concentrations, but the slope of the lines differed. The curves flatten when no visible precipitate is formed with the addition of ethanol-buffer mixture, the transmission at this stage being less than 100 per cent. This may be due to differences in the density of the test and blank solutions with a consequent difference in light focusing, or it may be due also in part to absorption of light by the gelatin solution.

In some cases it is desirable that the overall time for each estimation be short as, for instance, in the chromatography of enzyme solutions. The incubation time may then be reduced to 1 minute and the incubation temperature increased to 45°C. Under these conditions care must be taken that enzyme solutions are not inactivated prior to estimation and that there is no appreciable evaporation from the substrate solutions.

(*c*) *Estimation of Protease Activity Gravimetrically*

As the turbidimetric technique is not readily applicable to enzyme solutions containing considerable amounts of precipitable material or coloured components, a more general method for protease estimation has been developed.

(i) *Reagents.*—For the buffer substrate 20 g. of gelatin were added with stirring to a hot solution of glacial acetic acid (5 ml.) and 40 per cent. NaOH (10 ml.) in distilled water (900 ml.). The gelatin solution at approximately pH 5.0 was cooled and the pH adjusted with NaOH to the required value. The volume was adjusted to 1 l. and the pH checked. The precipitant was ethanol containing HCl or NaOH according to the pH of the gelatin used. When using a pH of 7.0 the precipitant contained 2.0 ml./l. of 5N HCl.

(ii) *Procedure.*—Aliquots (2 ml.) of buffer substrate in tared 15 ml. centrifuge tubes were equilibrated in a water-bath containing distilled water at the required temperature, and enzyme solution (0.5 ml.) was added. The tubes were shaken and incubated for 1 hour, then cold precipitant (10 ml.) was blown in to give rapid mixing. The tubes were allowed to stand in an ice bath for 15 minutes for completion of flocculation and were then centrifuged at 3000 r.p.m. in an angle centrifuge for 10 minutes. The clear supernatant was discarded, and the tubes inverted on absorbent paper for 5 minutes. They were then dried at 90-100°C. for 4 hours, cooled, and weighed. A control test was performed for each enzyme solution after heating in a boiling water-bath for 10 minutes. The difference between the weight of precipitate in the test and control tubes is a measure of the amount of gelatin rendered soluble under the conditions of the test. Since the accuracy attained using this method depends largely on accuracy in pipetting, it is advisable to hold the buffer substrate solution at 40°C. during pipetting. Increasing the time of standing before centrifuging, or the time of drying, has been shown to have no measurable effect on the results.

In Figure 7 the relationship between enzyme concentration and the amount of gelatin rendered soluble is shown for the crude protease of *A. oryzae*,

crystalline trypsin (Armour), and crystalline chymotrypsin (Armour), the enzyme solutions being prepared in 0.1M phosphate buffer at pH 7.0. With the mould protease, and to a lesser extent with the other enzymes, the relationship is approximately linear for low enzyme concentrations, and it is possible therefore to obtain a curve relating the amount of enzyme present to the experimental values. The unit of enzyme activity is taken to be that causing a reduction of 1 mg. in the precipitable gelatin at low enzyme concentrations. The curves of Figure 7 are calibration curves obtained in this way.

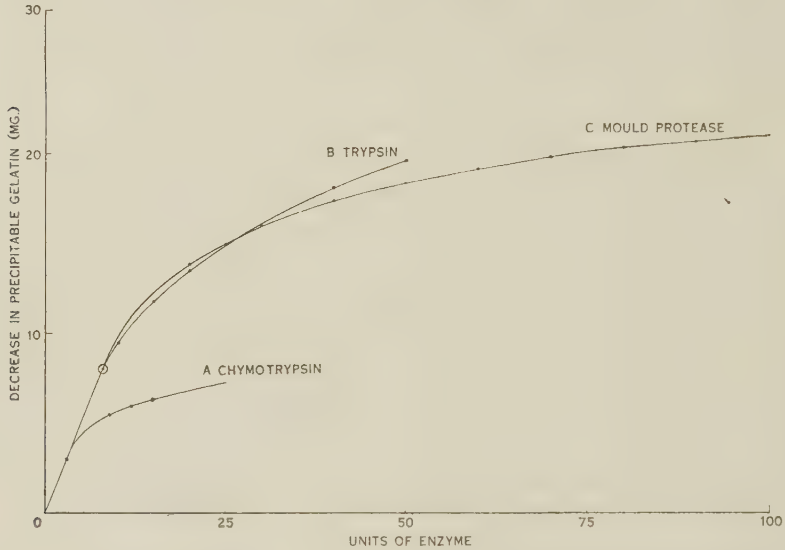

Fig. 7.—Calibration curves of the gravimetric estimation of protease activity. The relation between gelatin hydrolysed (mg.) and enzyme concentration for *A*, chymotrypsin; *B*, trypsin; and *C*, crude mould protease.

As would be expected, different samples of gelatin provide slightly different dilution curves for each enzyme solution. The dilution curves for the mould protease using Davis gelatin and Difco gelatin are shown in Figure 8 to be similar in shape, but there is a slight difference in absolute values. In experiments using this method a bulk stock of gelatin is therefore used, all calibration curves having been obtained with this material. It is possible to incorporate reducing agents or traces of metal ions in the buffer substrate where necessary.

The high buffer capacity of acetate at the pH of maximum precipitation of the gelatin used provides adequate protection against changes in pH as the result of addition of enzyme solutions of high buffer capacity and widely varying pH. When aliquots (2 ml.) of gelatin substrate solution at pH 7.0 were precipitated with aliquots (10 ml.) of ethanol containing different amounts of 5N HCl, maximum precipitation occurred with 1.8 ml. HCl per litre, but the variations in the amounts of gelatin precipitated were within experimental

error over the range 1.3-2.5 ml. HCl per litre. A titration curve of the buffer-substrate solution may be used to determine the amounts of acid or alkali required in the precipitant for maximal precipitation of the substrate as the pH of the buffer substrate varies from the point of maximum precipitation.

The foregoing method may be adapted to use the colorimetric biuret test, the gelatin precipitate being dissolved in water and estimated by the procedure for biuret estimation of proteins (Robinson and Hogden 1940).

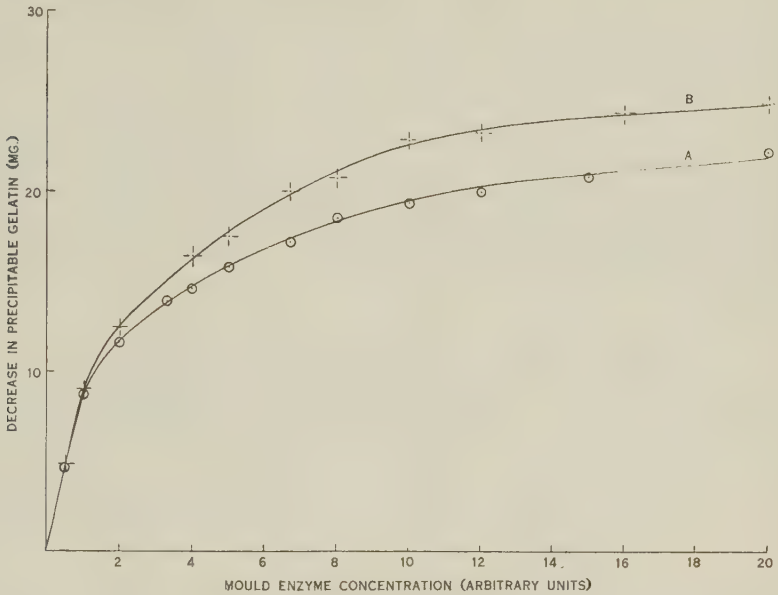

Fig. 8.—Dilution curves obtained for mould protease using *A*, Davis gelatin and *B*, Difco gelatin.

(*d*) *Comparison of Sensitivity of Methods of Protease Estimation*

In order to compare the sensitivity of these techniques for protease estimation with other well-known methods, solutions of crystalline trypsin, chymotrypsin, and pepsin (Armour); crystals containing protease from *A. oryzae* (Crewther and Lennox 1950); and non-crystalline papain (Parke-Davis Products) were diluted 1/10, 1/100, 1/1000, and 1/10,000 and estimated by the viscosity reduction of gelatin (Lennox and Ellis 1945), haemoglobin digestion (Anson 1938), acetone titration (Linderstrøm-Lang 1927), and by the gravimetric and turbidimetric techniques. The results shown in Table 1 indicate that the last two methods compare favourably with other methods of protease assay.

IV. Discussion

The fact that a straight-line relationship can be obtained between log light transmission and gelatin concentration for solutions of gelatin precipitated with ethanol suggests that the mean micelle size of the precipitated gelatin does

not vary appreciably with gelatin concentration. Bischoff and Desreux (1951) have demonstrated a similar constancy of mean micelle size for precipitated polymers, as the concentration of precipitant varies.

The two methods for protease estimation described in Section III are both based on the familiar principle of estimating the amount of substrate rendered non-precipitable as a result of enzyme action. When using gelatin as substrate, this involves the following considerations.

High molecular weight gelatin, which has been carefully prepared, can be readily broken down by boiling in water at physiological pH into smaller units, which are also present in considerable amount in commercial gelatin preparations. It is possible therefore that the high molecular weight components of commercial gelatin will comprise smaller units linked by readily hydrolysable peptide bonds, which may be more susceptible to the attack of certain proteases than are peptide bonds linking other amino acid residues. It is possible also that certain hydrogen bonds present in the larger components are absent from the degradation products.

TABLE 1

COMPARISON OF SENSITIVITY OF METHODS OF PROTEASE ASSAY

	Maximum Dilution Showing Significant Activity				
Enzyme	Viscosity Reduction of Gelatin	Haemoglobin Digestion	Acetone Titration	Gravimetric Method	Turbidimetric Method
Trypsin 0.5 mg./ml.	1/100	1/100	1/10	1/1000	1/100
Chymotrypsin 0.5 mg./ml.	1/100	1/10	1/10	1/100	1/10
Pepsin 0.5 mg./ml.	1/1	1/100	—	1/10	—
Mould protease 0.3 mg./ml.	1/100	1/100	1/10	1/1000	1/100
Papain 2.0 mg./ml.	1/100	1/10	1/10	1/100	1/10,000

It is to be expected therefore that, for any one enzyme, the sensitivity of the protease methods described above will depend on the concentration of ethanol used and the conditions of precipitation. Thus enzymes acting preferentially on components of the gelatin having molecular weights such that they are precipitated under the experimental conditions but may be rendered soluble by the hydrolysis of a single peptide bond, will be more readily detected than enzymes acting on the larger or smaller components of the gelatin, and therefore causing little decrease in the precipitable material present. It might be expected therefore that the gravimetric and turbidimetric methods would show

variable sensitivity to different proteases, as the threshold of molecular weight is different in the two methods. This is to some extent demonstrated in Table 1 where considerable variation is shown in the sensitivity of the two methods to the action of various proteases. Similarly the viscosity reduction of gelatin, which is probably related particularly to hydrolysis of the higher molecular weight components of gelatin, need not necessarily be sensitive to the same enzymes as the gravimetric method, which is concerned chiefly with the lower molecular weight fraction. Evidence that this is so will be presented in a later publication.

It will be appreciated that the turbidimetric method is limited to a rather narrow range of pH, it being necessary to vary the substrate concentration according to the pH used. The nearer the pH approaches the isoelectric point of the gelatin used, the lower will be the maximum concentration of gelatin it is possible to use. On the other hand, the sensitivity of the method would increase accordingly. At high pH the range of transmission values corresponding with 0-2 per cent. gelatin would be limited to higher values, and it should be possible to maintain sensitivity by using undiluted ethanol as precipitant.

The use of gelatin as substrate for protease estimation has the disadvantage that no standard preparation of gelatin can be obtained. On the other hand, its high solubility and the ease with which it is attacked by proteinases without prior denaturation are points in its favour. The process of denaturing a pure protein such as haemoglobin probably introduces substrate heterogeneity. The use of a substrate requiring no denaturation also avoids the possible effect of the denaturant on the enzyme itself.

V. Acknowledgment

The author wishes to thank Mrs. Joyce G. McIntyre and Mr. F. P. Murphy for technical assistance.

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INHERITANCE OF SPOTTED WILT RESISTANCE IN THE TOMATO

I. IDENTIFICATION OF STRAINS OF THE VIRUS BY THE RESISTANCE OR SUSCEPTIBILITY OF TOMATO SPECIES

By K. W. FINLAY*

[*Manuscript received February* 25, 1952]

Summary

Strains of the tomato spotted wilt virus were separated from field-infected material and purified with a range of solanaceous species. These strains were classified into four groups, Tip Blight (TB), Necrotic (N), Ringspot (R), and Mild (M), according to the symptoms developed by susceptible tomato hosts.

Strains within these groups were identified by the resistance or susceptibility of the five tomato types *Lycopersicon peruvianum*, *L. pimpinellifolium*, and *L. esculentum* var. Rey de los Tempranos, Pearl Harbour, and Manzana. This gave a total of 10 distinct strains.

It is claimed that this method of classifying the strains will facilitate the identification of genes for resistance to spotted wilt, so that their mode of inheritance can be studied.

A possible explanation of the variable resistance of the varieties Rey de los Tempranos, Pearl Harbour, and Manzana is given.

I. INTRODUCTION

The spotted wilt disease of the tomato has been the subject of extensive investigation in Australia and America for many years. Best and Samuel (1936*a*, 1936*b*) and Bald and Samuel (1934) made artificial inoculation a standard technique in the study of this virus when they showed that the infectivity of the virus was considerably prolonged by maintaining the extracted infected plant sap at pH 7.0, and by using a reducing agent, such as sodium sulphite.

Norris (1946) demonstrated the existence of five strains of the virus, which he separated from naturally occurring complexes. He named these strains Tip Blight (TB), Necrotic (N), Ringspot (R), Mild (M), and Very Mild (VM). The variability in the symptoms on tomatoes was shown to be caused mainly by the number and concentration of the individual strains comprising the field complexes.

In the search for tomato varieties with genetic resistance to the tomato spotted wilt (T.S.W.) virus, Dr. D. R. Porter, of the University of California, selected a strain of *L. pimpinellifolium* with high resistance. It was found, however, that a linkage existed between the *L. pimpinellifolium* characters and resistance to T.S.W. In an attempt to break this linkage by back-crossing with

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Porter's strain of *L. pimpinellifolium,* Hutton (1949, 1951) reported the inheritance of resistance as being obscure but likely to be controlled by a polygenic system.

Smith (1944) found that the wild tomato *L. peruvianum* was immune to T.S.W. in the field and by the use of embryo culture he was able to procure crosses with *L. esculentum.* Norris (1946) confirmed the very high resistance of *L. peruvianum,* by inoculating it with his five strains and showing that only the Tip Blight strain was capable of producing even very mild symptoms.

Pearl Harbour, the first commercial variety resistant to T.S.W., was bred by Kikuta, Hendrix, and Grazier (1945) in Hawaii and derived its resistance from Porter's strain of *L. pimpinellifolium.*

Holmes (1948) found that the two Argentinian varieties, Rey de los Tempranos and Manzana, were resistant to T.S.W. in New Jersey, but susceptible in Hawaii and that Pearl Harbour lost much of its resistance when grown in New Jersey. This breakdown of resistance in different localities was assumed to be due to differences in the strain composition of the virus complexes.

Finlay (1951) showed that in Western Australia the varieties Pearl Harbour, Manzana, and Rey de los Tempranos were all susceptible, but that an F_1 hybrid between Pearl Harbour and Rey de los Tempranos had a very high field resistance.

These investigations suggested that there were several genes controlling the inheritance of resistance to T.S.W. and that the presence or absence of some of the strains of the virus in local complexes also had an effect on the resistance or susceptibility of varieties such as Pearl Harbour, Manzana, and Rey de los Tempranos.

The following investigation commenced in 1949, and was based on the hypothesis that there exists a complex of genes for resistance to T.S.W. virus, and that each of these genes controls resistance to one or more individual strains of the virus. To test the validity of this hypothesis it was necessary to separate as many strains of the virus as possible and to ascertain the resistance or susceptibility of tomato varieties and species to these strains.

II. Materials and Methods

(*a*) *Strain Separation*

Two complexes of T.S.W. virus, obtained from commercial tomato plants, were used as the original source of strains. The first complex induced typical Tip Blight symptoms on a plant of the variety Wanneroo Late in the field. The second complex, found on a plant of variety King Humbert, had a higher concentration of milder strains as typified by the mild mottling of young leaves followed by a delayed necrosis about 7 days after the appearance of the first symptoms.

(i) *Norris's Method.*—Norris (1946) gave a very full account of his methods of strain separation, using a range of solanaceous plants for their differentiation. These methods were followed in this investigation.

The virus strains were kept in an active state by hand inoculation every 3 weeks onto young tomato plants of the variety Potentate. The plant tissue used as a source of inoculum was selected when the first symptoms were most marked, at which stage they contain a high concentration of virus. The time between inoculation and the appearance of first symptoms, however, varied with the virus strain used.

Infected leaf tissue (1.0 g.) was ground in a mortar with 12.0 ml. of a buffer solution of pH 7.0 made up of 3 parts M/10 Na_2HPO_4 plus 2 parts M/10 NaH_2PO_4 together with 0.2 per cent. sodium sulphite as a reducing agent. Dusting of all plants with fine carborundum powder before inoculating was standard procedure. Inoculation was performed with a ground-glass spatula. All the plant material for the experiments was grown in an insect-proof glass-house.

From the two original complexes in Wanneroo Late and King Humbert four strains of the virus were isolated in a pure or almost pure form. They gave similar reactions to Norris's Tip Blight, Necrotic, Ringspot, and Mild strains. Norris's (1946) Necrotic strain had been isolated from a potato plant and not from tomato.

(ii) *Imported Strains.*—Through the cooperation of Dr. R. J. Best, tomato plants carrying three strains of T.S.W. virus were flown from the Waite Institute, South Australia. These strains Best and Gallus (1947) named A, B, and C in decreasing order of severity of symptoms developed by susceptible tomato plants. When tested these strains were found to produce host reactions similar to those caused by TB, N, and R described by Norris (1946).

(iii) *Possible New Strains.*—During 1951, in a field of 1000 segregating tomato hybrids and parent lines, many of which exhibited resistance to T.S.W., a line of Porter's strain of *L. pimpinellifolium* became badly infected with T.S.W. virus. As Porter's strain of *L. pimpinellifolium* had not previously been reported susceptible in the field, the severe strain of virus was isolated and appeared to be identical to TB strain on susceptible tomato hosts. In Porter's strain of *L. pimpinellifolium* the symptoms were a severe systemic necrosis on the lower leaves, gradually progressing up the stems, and finally killing the plants. This strain was called TB_2.

Several other strains were separated from material in this field, many plants being badly infected, but the strains appeared to be identical with those already isolated. All were maintained, however, for further testing.

(b) *Culture and Inoculation of Tomato Species used in Strain Differentiation*

The tomato varieties and species selected for differentiating strains of T.S.W. were *L. peruvianum,* Porter's strain of *L. pimpinellifolium,* and the *L. esculentum* varieties, Rey de los Tempranos, Pearl Harbour, and Manzana. All these exhibit resistance to T.S.W. virus in different areas of the world.

Several hundred plants of these varieties were grown in 3-in. pots in an insect-proof glass-house under hot conditions (95-100°F.). This treatment produced hardy plants similar to field-grown ones. When 10-12 in. high these

plants were transferred to a basement room at a temperature of about 85°F., and illuminated by two banks of fluorescent lights (14 40-watt tubes per bank). Commercial "Daylight" and "Soft white" tubes were alternated to give a spectrum range. The banks of lights were lowered to within about 2 in. of the top of the plants, to give a light intensity of about 500 ft.-candles (Plate 1).

The plants were given 24 hours dark treatment before inoculation and from then on a 12-hour day until the trial was completed. These lighting and temperature treatments were given to standardize environmental conditions within reasonably close limits because different reactions had been noted under varying environmental conditions. Four leaves just below the growing tip were inoculated on each plant.

This experimental procedure was adopted in order to simulate as nearly as possible field conditions as experienced in Western Australia, because it was hoped that the knowledge gained from these experiments might be applied directly to practical breeding for resistance to the T.S.W. virus disease. The environmental conditions imposed on the experimental plants just prior to and following inoculation tended to favour the virus.

Because of space limitation under the banks of fluorescent lights, a series of trials were conducted over a period of about 12 months, under environmental conditions as nearly identical as possible.

A 9 by 9 latin square layout was employed, made up of three varieties inoculated with three strains and replicated nine times. The trials were conducted so that tomato types *L. peruvianum*, Porter's strain of *L. pimpinellifolium*, and Rey de los Tempranos were inoculated by three virus strains in a latin square design, and placed under one bank of fluorescent tubes. Tomato types Rey de los Tempranos, Pearl Harbour, and Manzana were inoculated with the same three strains and placed under the other bank of fluorescent tubes.

At the end of each trial (usually 30-40 days) further plants were inoculated with two of the strains used in the previous trial plus one new strain. This procedure was continued until each variety had been inoculated at three different intervals of time, by the same virus strain, i.e. 27 plants of each variety had been inoculated with each strain, except variety Rey de los Tempranos, which was inoculated with each strain 54 times, being duplicated under each set of fluorescent lights (Table 1).

The necessity of conducting a series of trials with relatively small numbers of plants has tended to reduce the statistical precision of this experiment. By maintaining constant environmental conditions, plus employing standardized inoculum and inoculating technique, the error has been reduced to a minimum.

Although a fully susceptible variety of tomato was not included in the trials, the symptoms of all virus material were checked before and after the experiments on the fully susceptible tomato variety Potentate.

The reactions of the tomato varieties and species were classified into three categories:

Immune—plants that showed no reaction to the virus, even after two inoculations.

Resistant—plants exhibiting symptoms, but able to overcome the effect of the virus and continue growth free of the virus.

Susceptible—plants exhibiting symptoms, but unable to overcome the effect of the virus.

TABLE 1

NUMBERS OF IMMUNE (I), RESISTANT (R), AND SUSCEPTIBLE (S) PLANTS OF FIVE TOMATO SPECIES AND VARIETIES WHEN INOCULATED WITH 10 STRAINS OF T.S.W. VIRUS.

Tomato variety	*L. peruvianum*			*L. pimpinel-lifolium*			Rey de los Tempranos			Pearl Harbour			Manzana		
No. plants inoculated	27			27			54			27			27		
Plant reaction	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S
Strains of T.S.W. virus															
TB_1	1	26			26	1		54				27			27
								(3)*							
TB_2	27					27		54				27		27	
TB_3	27				27				54		27				27
					(3)						(27)				
N_1	27				27				54		27				27
					(3)						(2)				
N_2	27				27			54							27
R_1	27			27					54			27			27
R_2	27			27				54			27			27	
											(27)			(2)	
R_3	27			27			53	1				27		27	
												27		(1)	
M_1	27			27			53	1		27					27
M_2	27			27			54			27				27	
														(27)	

* The figures in parentheses indicate the number of resistant plants with systemic infection in addition to local lesions on inoculated leaves.

III. RESULTS

By using the method of strain differentiation outlined, 10 distinct strains of the T.S.W. virus were identified. The strains were named TB_1, TB_2, TB_3, N_1, N_2, R_1, R_2, R_3, M_1, and M_2. Table 2 classifies the resistance or susceptibility of the five tomato types to these strains.

(*a*) *Strain TB_1*

This is Best's strain A, which gives a typical tip blight reaction on susceptible tomato varieties.

(i) L. peruvianum.—The development of numerous small, necrotic lesions was evident on 26 plants of this species. One plant showed no reaction to the virus even after reinoculation. The lesions did not enlarge or become systemic. This species was, therefore, rated as resistant to strain TB_1.

(ii) L. pimpinellifolium.—This developed necrotic local lesions with a gradual systemic spreading of the disease, but under the environmental conditions imposed by the experiment, 26 of the 27 plants outgrew the infection and appeared healthy after 35-40 days. One plant died. They were rated as resistant. It was noted that when this species was grown at lower temperatures and the plants were much "softer" they were quite susceptible.

(iii) *Rey de los Tempranos.*—This variety gave a reaction typically the same as *L. pimpinellifolium* with the disease spreading gradually up the stems. The plants appeared, however, to be able to localize the disease eventually and grow away quite normally. This strain had no effect on the setting or quality of fruit even in the diseased portion of the plant, and the variety was rated as resistant.

(iv) *Pearl Harbour and Manzana.*—These appeared to be quite susceptible, the inoculated leaves exhibiting necrotic local lesions after 6-7 days, followed by a rapid systemic spreading to the upper portion of the plant, causing darkened stems that collapsed and died rapidly. The plants were all dead within 21 days. Both of these varieties were rated as susceptible.

TABLE 2

REACTION OF FIVE TOMATO TYPES TO 10 STRAINS OF SPOTTED WILT

Tomato Differentials	T.S.W. Virus Strains									
	Tip Blight Group			Necrotic Group		Ringspot Group			Mild Group	
	TB_1	TB_2	TB_3	N_1	N_2	R_1	R_2	R_3	M_1	M_2
L. peruvianum	R	I	I	I	I	I	I	I	I	I
L. pimpinellifolium	R	S	R	R	R	I	I	I	I	I
Rey de los Tempranos	R	R	S	S	R	S	R	I	I	I
Pearl Harbour	S	S	R	R	S	R	S	S	I	I
Manzana	S	R	S	S	S	S	R	R	S	R

I = immune; R = resistant; S = susceptible.

(*b*) *Strain* TB_2

This strain was isolated from plants of Porter's strain of *L. pimpinellifolium* infected in the field.

(i) L. peruvianum.—This appeared to be quite immune to this strain, showing no lesions on inoculated leaves, and it was rated as immune.

(ii) L. pimpinellifolium.—Definite yellow, local lesions appeared after 7-8 days but they rapidly became necrotic on inoculated leaves. After about 14 days the systemic spread of the disease was evident, causing a diffuse mottling on the younger leaves, which later developed into an interveinal necrosis and finally either shrivelled or abscissed. After an average of about 35 days the virus had progressed up the stems and killed the whole plant. Rated as susceptible.

(iii) *Rey de los Tempranos and Manzana.*—These were similar to *L. pimpinellifolium* in reaction to this strain, but the symptoms were less severe, only odd leaflets exhibiting a necrosis developing from the diffuse mottling. All the plants grew away and apparently completely recovered after about 35-40 days. These varieties were rated as resistant.

(iv) *Pearl Harbour.*—The reaction was typical of normal Tip Blight, the plants being killed in 14-16 days after inoculation; rated as susceptible.

Owing to the typical Tip Blight reaction of this strain on susceptible plants and Pearl Harbour it must be classed in the TB group, even though its reaction to *L. pimpinellifolium* is atypical.

(*c*) *Strain* TB_3

The strain was isolated from a plant of the variety Wanneroo Late growing in the field.

(i) L. peruvianum.—This gave no reaction on any of the 27 plants inoculated and was rated immune.

(ii) L. pimpinellifolium.—After 7-8 days necrotic local lesions appeared on 25 of the 27 plants that were inoculated and after 14-16 days these were followed, in three plants, by a mild chlorotic systemic infection on three to five leaves above those inoculated. No spread of the disease was evident up to 30 days after inoculation. After 10 days the other two plants were reinoculated and produced substantially the same symptoms as the other 25, indicating a "miss" in inoculation. This variety was rated as resistant.

(iii) *Rey de los Tempranos.*—This reacted to TB_3 in a manner similar to its behaviour with strains TB_1 and TB_2 but instead of the plants gradually overcoming the virus, the systemic spread gradually built up, producing a chlorotic mottle followed rapidly by severe interveinal necrosis and some necrotic spotting on the very young leaves. The plants died after 25-30 days and were rated as susceptible.

(iv) *Pearl Harbour.*—After 6-7 days local necrotic lesions appeared and these were followed by systemic necrotic spotting and stunting of the growing tip. New growth completely free of disease formed 28-32 days later. No virus infection could be produced on *Nicotiana glutinosa* from sap extracted from the new, healthy shoots and the plants were rated as resistant.

(v) *Manzana.*—Local lesions formed after 5-6 days with systemic necrosis in the stems and leaves above and sometimes below inoculated leaves. After 16-18 days the plants were dead and were classed as susceptible.

(*d*) *Strain* N_1

This strain was obtained from a plant of the variety King Humbert grown in the field.

(i) L. peruvianum.—No symptoms were evident on any of the inoculated plants, which were rated as immune.

(ii) L. pimpinellifolium.—After 12-14 days indefinite yellow spots appeared surrounded sometimes by fine concentric yellow rings. With three plants these spread and after about 28 days the systemic infection had caused slight vein clearing and curling down of the young leaves, eventually stunting the growth. New growth on these three plants developed without symptoms and as the other 24 plants showed no systemic spread the variety was rated as resistant.

(iii) *Rey de los Tempranos.*—Indefinite chlorotic spotting, which later became necrotic, appeared after 10-12 days on all the plants. Systemic vein clearing was also evident in all plants with curling down of leaves and stunting of the growing tip whilst necrosis was later evident in the systemically affected areas. New growth developed with similar symptoms and produced a few fruit, which were small and showed a distinct yellow ring pattern, causing the variety to be classed as susceptible.

(iv) *Pearl Harbour.*—Very mild, indefinite, yellow spotting developed, which spread slowly to form a diffuse mottling and an occasional sharply defined green "island." Only two plants showed signs of systemic infection, and then only on two or three leaves above those inoculated. The growing tip and new growth developed without symptoms and the variety was rated as resistant.

(v) *Manzana.*—This gave substantially the same reaction as Rey de los Tempranos except that the systemic spread was generally more rapid and severe, much more necrosis being evident on young leaves. The rating was susceptible.

(*e*) *Strain* N_2

This strain gives the same reaction and is identical with Best's strain B.

(i) L. peruvianum.—No symptoms were evident on any plants, which were rated as immune.

(ii) L. pimpinellifolium.—Yellow ringspot lesions appeared on inoculated leaves after 7-8 days and these gradually enlarged, becoming necrotic around the circumference. There was no systemic spread of the disease, giving a resistant rating.

(iii) *Rey de los Tempranos.*—Indefinite, yellow mottling and ringspot lesions on inoculated leaves after 7-8 days, a necrotic pattern becoming evident on the outer edges of the chlorotic areas after about 20 days. There was no systemic spread of the disease, which rated the variety as resistant.

(iv) *Pearl Harbour and Manzana.*—These two varieties reacted similarly to this strain, local chlorotic lesions appearing after 7-8 days, which later became necrotic at the circumference of the lesions to produce a necrotic pattern, which caused leaves to shrivel and die. Sixteen to 20 days after inoculation bronze spot-

ting became evident on the young leaves and also a chlorotic mottling with some necrosis on some of the leaves below those inoculated. The upper leaves wilted and shrivelled slowly.

After about 40 days, weak new growth appeared on five plants of Pearl Harbour and four plants of Manzana, but even this exhibited a stunting and diffuse mottle on the leaves. Two young shoots also had some bronze spotting and the varieties were rated as susceptible.

(*f*) *Strain R_1*

The strain is equivalent to Best's strain C.

(i) L. peruvianum.—No symptoms were evident up to 30 days after inoculation, giving an immune rating.

(ii) L. pimpinellifolium.—No symptoms appeared up to 30 days. Rated immune.

(iii) *Rey de los Tempranos.*—A chlorotic mottle appeared after about 20 days, varied slightly from plant to plant, but it was either in the form of a fine, concentric, yellow line pattern, or a diffuse mottling with a few sharply defined green "islands."

No necrosis was evident on any of the plants at any stage of infection. The younger leaves were stunted and rugose, having a tendency to curl downwards. Rated as susceptible.

(iv) *Pearl Harbour.*—Vein clearing and downward curling of the inoculated leaves was followed by the appearance of a diffuse mild mottle on two or three leaves above those inoculated. In no plants did the disease become fully systemic, the plants growing on quite normally after a slight reduction in the growth rate for between 10 and 30 days. Rated resistant.

(v) *Manzana.*—Symptoms similar to those produced in Rey de los Tempranos (iii) except that five plants exhibited a marked interveinal necrosis on some young leaves, which subsequently shrivelled and died. Rated susceptible.

(*g*) *Strain R_2*

This strain was isolated from a tomato plant of the variety King Humbert.

(i) L. peruvianum.—No symptoms evident after inoculation. Rated immune.

(ii) L. pimpinellifolium.—No symptoms. Rated immune.

(iii) *Rey de los Tempranos.*—Vein clearing and curling down of inoculated leaves occurred after 8-9 days and these leaves yellowed and fell off. On only two plants did systemic infection manifest itself as a slight vein clearing on some of the younger leaves. Rated resistant.

(iv) *Pearl Harbour.*—Vein clearing and curling down of inoculated leaves occurred after 7-8 days. The plants became dwarfed with chlorotic, rugose,

and distorted leaves. Some younger leaves developed a bronze pattern and others a definite concentric line ring pattern. New growth exhibited a strong mottle and distortion with sharply defined, raised, green "islands." Rated susceptible.

(v) *Manzana.*—This variety reacted in a similar manner to strain R_2 as did Rey de los Tempranos. Rated resistant.

(*h*) *Strain R_3*

This strain was isolated from a complex infecting a hybrid tomato plant of genetic constitution resulting from a tetraploid hybrid Pearl Harbour × Rey de los Tempranos crossed with a diploid Manzana.

(i) L. peruvianum, L. pimpinellifolium, *and Rey de los Tempranos.*—No symptoms appeared on any of these plants after inoculation, except local lesions on inoculated leaves of one plant of Rey de los Tempranos. Rated immune.

(ii) *Pearl Harbour.*—A diffuse mottle appeared after 8-9 days on inoculated leaves. The systemic symptoms comprised a vein clearing with down-curling of the young leaves, stunting of the plants, and the appearance of sharply defined mottling with some interveinal necrosis and rugosity of the leaves. The new growth was mottled and rugose. Rated susceptible.

(iii) *Manzana.*—A vein clearing and down-curling of inoculated leaves was evident after about 7-8 days and this was followed by a slight mottling of the young leaves, but this gradually disappeared, leaving apparently healthy plants after about 28-30 days. Rated resistant.

(*i*) *Strain M_1*

This strain was obtained from plants of *L. esculentum* var. King Humbert infected with a mild complex in the field and also from *L. pimpinellifolium.*

(i) L. peruvianum, L. pimpinellifolium, *Rey de los Tempranos, and Pearl Harbour.*—Symptoms were only evident on inoculated leaves of one plant of Rey de los Tempranos. Rated immune.

(ii) *Manzana.*—Slight vein clearing on the inoculated leaves became evident after about 12 days. A systemic mild mottling of young leaves with slight rugosity appeared after 21-25 days. No effect on the vigour of the plants was noticed, but slight traces of the mottle were evident for a considerable time. On six of the plants the fruit showed faint yellow ring markings. Rated susceptible.

(*j*) *Strain M_2*

This strain was obtained from plants of several different tomato varieties. All the isolates gave the same reaction, so that all except that from one line were discarded.

(i) L. peruvianum, L. pimpinellifolium, *Rey de los Tempranos, and Pearl Harbour.*—No symptoms became evident after inoculation; the varieties were rated immune.

(ii) *Manzana.*—A slight mottling appeared on the young leaves of all plants but this quickly disappeared. It had no effect on the vigour of the plants, which were rated as resistant.

IV. Discussion

The reaction of genetically different tomato hosts to individual strains of the spotted wilt virus has enabled the number of known strains to be increased to 10. Norris (1946) was able to isolate a very mild strain, which has not as yet been accomplished in this project. It is reasonable then to assume that there is at least one more strain in existence, apart from the 10 described in this paper.

It is proposed to classify these strains by grouping them firstly according to the symptoms they produce on susceptible tomato hosts, e.g. Tip Blight (TB), Necrotic (N), Ringspot (R), and Mild (M). The strains within any one group are further differentiated by the resistance or susceptibility of the five selected tomato types to them, and are numbered as identified, e.g. TB_1, TB_2, etc. This method of classification is simple and will accommodate further strains as they appear.

A possible explanation of the results gained by Holmes (1948) and Kikuta, Hendrix, and Grazier (1945) is offered by this work. It is suggested that the Hawaiian T.S.W. complex consists of all or some of the strains TB_3, N_1, R_1, M_1, and M_2, accounting for the high resistance of Pearl Harbour in that locality. The New Jersey complex, however, may consist of any number of the strains TB_1, TB_2, N_2, R_2, R_3, M_1, and M_2. This would account for the apparent resistance of Rey de los Tempranos and Manzana, and could also explain the apparent higher resistance of Rey de los Tempranos as compared with Manzana. The latter variety is susceptible to strains TB_1 and N_2, which may occur only occasionally in the complexes, causing the resistance of Manzana but not of Rey de los Tempranos to break down.

It is not known whether the strains of the spotted wilt virus present in other countries are identical with those found in Australia but judging by the susceptibility of tomato varieties found to be resistant in other parts of the world it appears likely that a greater range is present in this country.

Although specific resistance or susceptibility reactions are obtained by genetically different tomato plants, these cannot be used in strain purification. Norris (1951) obtained evidence of synergism of strains of spotted wilt, by showing that although Mild and Very Mild strains are present with Ringspot strains in the naturally occurring virus complexes found in potatoes, the former are unable to invade the potato in their pure form. Further evidence of a synergism appeared during the experiments described in this paper, when *L. pimpinellifolium* became infected with T.S.W. in the field. A virulent strain

TB_2, which apparently accounted for the breakdown of resistance, was isolated from this complex, but strains R_2 and M_1 were also obtained from this plant. *L. pimpinellifolium* is immune to the pure forms of strains R_2 and M_1.

The strain reactions given in Table 2 demonstrate the benefits to be gained by perseverence with hybrids involving *L. peruvianum* as a source of resistance. *L. pimpinellifolium,* however, is susceptible to strain TB_2. It is therefore inadvisable to use this species in breeding work, at least in Western Australia, where other strains are able to infect the plants if they are present in a complex with strain TB_2.

Finlay (1951) noted that the F_1 hybrid Pearl Harbour × Rey de los Tempranos had a very high resistance to T.S.W. complexes in the field, even though the two parent varieties were susceptible. The resistance possessed by each of the parents to some of the individual strains of the virus, as shown in Table 2, was transferred to the F_1 hybrid additively, producing resistance to a wide range of strains as may be found in field complexes.

It is proposed to use the methods described in this paper in an attempt to elucidate the number and mode of inheritance of the genes controlling resistance to T.S.W. The F_1 and F_2 populations of crosses in all combinations of Porter's strain of *L. pimpinellifolium,* Rey de los Tempranos, Pearl Harbour, and Manzana are being tested with the 10 strains so far identified. This work will be published later as Part II of this study.

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Fluorescent lights used to control the light intensity and length of day on experimental plants after inoculation.

THE PHYSIOLOGY OF GROWTH IN APPLE FRUITS

III. CELL CHARACTERISTICS AND RESPIRATORY ACTIVITY OF LIGHT AND HEAVY CROP FRUITS

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Summary

Cell size, total and protein nitrogen, and preclimacteric respiration have been studied for light and heavy crop fruit of certain Tasmanian-grown apple varieties. Differences in size of fruit from light and heavy crops have been shown to be due mainly to differences in cell size rather than in cell number. Respiration per cell, protein nitrogen per cell, and cell volume were closely intercorrelated but respiration per unit protein is greater in light crop fruit than in heavy crop.

It is suggested that the more rapid senescence and susceptibility to storage disorder of light crop fruit may be related to its higher respiration per unit protein. Though protein synthesis keeps pace with cell enlargement, the respiration per unit protein increases with cell size.

No consistent correlation was found between cell characters of a variety, such as cell size and cell number, and physiological characters such as period or date of maturation.

Attempts to raise the mean fruit size without impairing keeping quality are most likely to succeed if cell number per fruit is increased and cell size is kept small.

I. INTRODUCTION

It has long been realized that apples from light crops have a higher susceptibility to cool-storage disorders than those from heavy crops. Carne and Martin (1938) have demonstrated a high degree of correlation between the mean size of the fruit on a tree and the incidence of storage disorders and certain physiological characteristics of the fruit. More recently, Smock (1949) showed that, in gas storage, light crop fruit was more susceptible to brown core and Martin and Carne (1950) demonstrated a similar relation for brown heart. Studies of the differences between light and heavy crops have not been of much assistance in efforts to analyse and improve storage behaviour.

This difference is obviously bound up with differences in respiratory behaviour, but respiration, on a unit fresh weight basis, has shown no consistent difference between large and small fruit (Smock and Gross 1950), and light and heavy crops (Martin, unpublished data). The data given by Hulme (1951) suggested that, in a light crop year (1937), respiration per unit protein was higher than in normal years.

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Similarly, though apple varieties differ widely in characteristics such as mean fruit size for the variety, rate of maturation, storage behaviour, chemical characteristics, and dessert quality, the physiological basis of these differences, and their relation to susceptibility to disorder, have been little studied. Smith (1940, 1950), using English varieties, showed that varietal fruit size was determined by the amount of cell division after pollination, that apples of different varieties differed widely in cellular structure, and that differences in storage behaviour were correlated with cell size. Hulme (1951) considered that in normal years, respiration per unit protein was constant for each variety and was higher in dessert than in culinary varieties.

Bain and Robertson (1951) and Robertson and Turner (1951) have investigated the within-tree relation between fruit size, cell size, cell number, total and protein nitrogen, and respiration rate. This report applies the methods of Robertson and his co-workers to the study of light and heavy crop fruit and extends the work of Smith (1940, 1950) and Hulme (1951) to varieties growing in Tasmania.

II. Materials and Methods

Of the many varieties grown in Tasmania, those whose characteristics are given in Table 1 were selected to provide a suitable range of varietal characters. For crop contrast within a variety a search was made for two adjacent trees of similar growth form and vigour but of contrasting crop. Satisfactory pairs were found for Cleopatra, Cox, Crofton, Democrat, Delicious, Granny Smith, Ribston, Sturmer, and Tasman Pride. For the other varieties fruit was available as follows: Alfriston, trees in different orchards but on the same soil type; Alexander, Geeveston Fanny, and Prince Alfred, one tree of average crop only; Golden Delicious, trees irrigated and crops irregular; Jonathan, crop contrast not marked; and Worcester, no trees of contrasting crop and two adjacent trees of average crop but contrasting fruit size due to different growing conditions used.

From each tree a sample of 20 fruits was selected according to a procedure designed to produce random sampling at a date judged to be close to commercial maturity and before the onset of the climacteric. In Cox, Cleopatra, and Democrat a second picking from the same trees was made 2 weeks later, and with Granny Smith another picking from another source was made 2 weeks later. Each fruit was weighed and measured.

Of these 20 fruits, four of approximately mean size for the sample were selected for cell measurements and 10 were used to determine the rate of respiration at 25°C. by the Pettenkofer method, taking the mean rate for the 8-hour period 40-48 hours after picking. This 10 and the remaining six were later examined for pressure by penetrometer, starch pattern by the starch-iodine method, and soluble solids of juice by refractometer. The mid-cortical tissue was then sliced, dried at 60°C. in an air oven, ground, and stored at 1°C. in sealed jars for analysis.

The four fruits for cell size measurements were halved equatorially and from the two positions on opposite sides of the fruit sections were cut from

the mid cortex parallel to the equator. These were fixed and stored in formalin-alcohol (6 ml. formalin in 100 ml. 78 per cent. alcohol). Four sections from each fruit were mounted and 25 cells from each were projected and cell

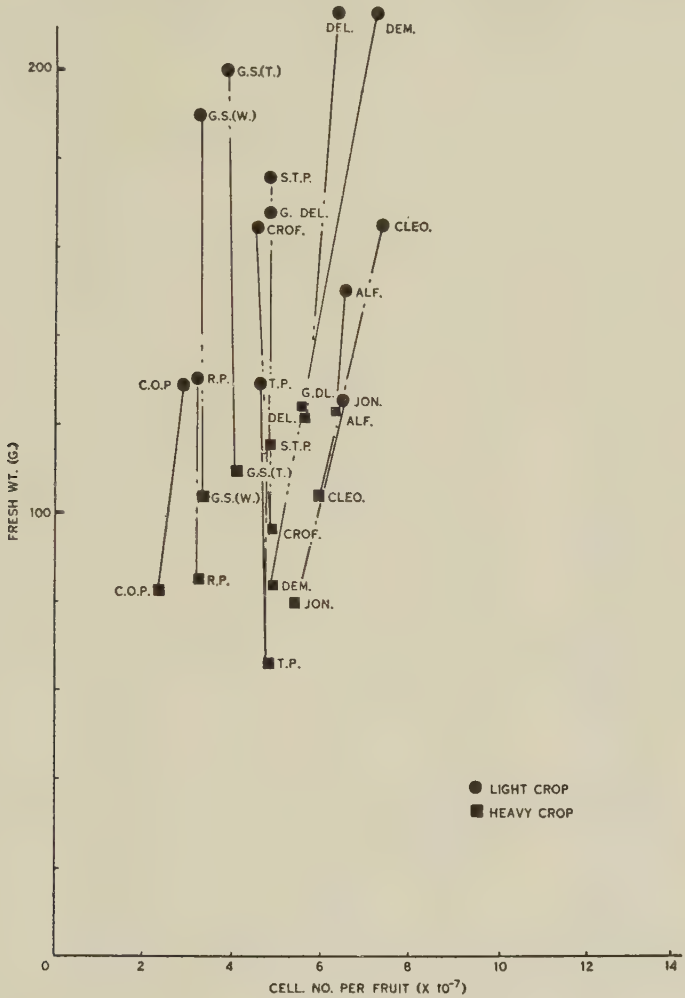

Fig. 1.—Mean weight and cell number per fruit of light and heavy crops of different varieties. Abbreviations used in this and later figures: Alex., Alexander; Alf., Alfriston; Cleo., Cleopatra; C.O.P., Cox's Orange Pippin; Crof., Crofton; Dem., Democrat; Del., Delicious; Eng., English material; G.F., Geeveston Fanny; G. Del., Golden Delicious; G.S., Granny Smith; Jon., Jonathan; P.A., Prince Alfred; P.D.N., Pomme de Neige; R.P., Ribston; S.T.P., Sturmer; T.P., Tasman Pride; W.P.M., Worcester.

volumes and surface areas computed by the method described by Bain and Robertson (1951) and Robertson and Turner (1951). Care was taken to avoid regions of small cells near the vasculars.

TABLE 1

VARIETY CHARACTERISTICS

Variety		Size*	Flowering Order†	Maturation Order‡	Full Bloom to Maturity (days) (tentative only)	Susceptibility to Breakdown§	Susceptibility to Pit§	Storage Life††	Surface Colour**	Acidity
Alexander	C¶	4	3	1	120	2	2	2	2	Medium
Alfriston	C	3	3	1	120	1	2	2	1	Medium
Cleopatra	C	3	2	3	150	1	4	3	1	Medium
Cox	D	1	2	2	140	4	4	1	2	High
Crofton	D	1	2	5	170	1	1	4	3	Low
Democrat	D	4	3	5	170	1	1	4	4	Low
Delicious	D	3	2	4	160	3	1	3	3	Medium
Geeveston Fanny	D	2	2	2	150	3	1	2	2	Low
Golden Delicious	D	3	3	4	150	2	1	2	1	Low
Granny Smith	D	3	2	5	170	1	2	4	1	High
Jonathan	D	2	2	3	150	3	1	3	3	Medium
Pomme de Neige	D	1	1	2	130	3	2	1	2	Low
Prince Alfred	C	5	1	2	140	2	1	2	2	Medium
Ribston	D	3	1	2	130	4	4	1	2	High
Sturmer	D	3	2	5	180	3	3	4	1	High
Tasman Pride	D	3	3	3	150	3	2	2	4	Medium
Worcester	D	2	4	1	110	1	1	2	4	Low

* Range of mean size 2¼-3¼ in.

† Range of mean date 3 weeks.

‡ Range of mean date 3 months.

§ 1, Not susceptible; 2, susceptible in light crops only; 3, susceptible; 4, very susceptible.

†† Very tentative for average crops only and without reference to breakdown.

** 1, No pigment; 2, $<$ 25%; 3, 25-50%; 4, $>$ 50%.

¶ D, dessert; C, culinary only.

Cell number was calculated from fruit weight and cell volume, assuming a specific gravity of 1.1 for the cell, following Smith (1940).

For varietal comparisons, data from Table 1 are used and the mean figure from the light and heavy crop or the figure for the one tree of average crop, when the light and heavy contrast is not available, is taken as representing the mean for the variety.

TABLE 2

VARIETAL CELL CHARACTERISTICS

Variety	Date of Picking	No. of Fruit on Tree	Mean Fresh Weight per Fruit (g.)	Cell No. per Fruit ($\times 10^{-5}$)	Respiration /Cell (mg. CO_2/hr. $\times 10^{-11}$)	R/P (mg. CO_2/g. protein N/hr. $\times 10^{-2}$)
Worcester	Feb. 13	375	92.8	479	439	1009
	Feb. 13	420	123.3	545	787	1182
Alfriston	Feb. 13	—	123.6	627	293	530
	Feb. 15	300	151.1	649	—	600
Prince Alfred	Feb. 15	500	317.4	1090	—	—
Pomme de Neige	Feb. 15	650	99.2	307	—	—
Cox—pick 1	Feb. 19	1000	83.0	236	1322	1421
	Feb. 19	400	130.2	280	1743	1760
Cox—pick 2	Mar. 4	—	98.7	246	825	738
	Mar. 4	—	147.0	285	1393	1280
Alexander	Feb. 22	274	143.8	870	—	—
Ribston	Feb. 26	—	85.4	327	786	952
	Feb. 26	—	130.6	321	1586	1438
Jonathan	Mar. 7	820	80.3	542	354	861
	Mar. 7	475	125.7	637	403	925
Cleopatra—pick 1	Mar. 7	900	107.2	593	435	847
	Mar. 7	470	166.3	729	475	910
Cleopatra—pick 2	Mar. 19	—	119.2	564	424	827
	Mar. 19	—	182.4	739	494	968
Tasman Pride	Mar. 14	750	66.4	481	302	703
	Mar. 14	450	130.1	455	503	694
Delicious	Mar. 26	680	125.1	548	573	1282
	Mar. 26	95	213.0	644	1357	1955
Golden Delicious	Mar. 26	670	124.3	553	591	1201
	Mar. 26	470	168.5	487	840	1157
Geeveston Fanny	Mar. 28	—	124.4	498	476	1094
Democrat—pick 1	Apr. 3	900	89.9	461	360	631
	Apr. 3	400	192.1	770	653	1066
Democrat—pick 2	Apr. 25	—	84.0	471	616	1101
	Apr. 25	—	214.1	725	1187	1680
Crofton	Apr. 16	930	97.2	478	519	910
	Apr. 16	175	164.8	447	1137	1055
Granny Smith—source 1	Apr. 16	470	104.4	327	575	711
	Apr. 16	120	191.3	333	1223	1014
Granny Smith—source 2	Apr. 25	500	110.6	412	1204	1559
	Apr. 25	350	200.6	387	2087	1625
Sturmer	Apr. 30	670	116.7	471	762	952
	Apr. 30	315	177.4	461	1718	1627

III. Results

The data are summarized in Table 2.

(*a*) *Cell Number per Fruit*

The difference in fruit size between light and heavy crops was a matter of cell size rather than cell number. As is illustrated in Figure 1 there was no significant difference in cell number between light and heavy crop fruit of the varieties Alfriston, Cox, Crofton, Golden Delicious, Granny Smith, Ribston, Sturmer, and Tasman Pride, and in the others, Cleopatra, Democrat, Delicious, and Jonathan, difference in fruit size was due more to cell size than to cell number.

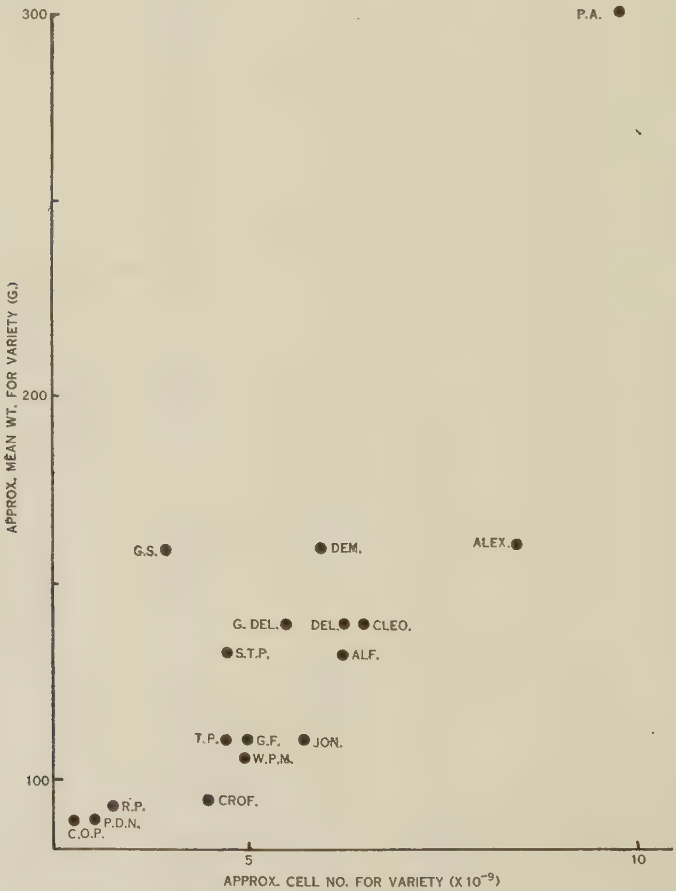

Fig. 2.—Relation between approximate mean fruit weight and mean cell number per fruit for different varieties.

There is probably a characteristic cell number for each variety and if the data available are used to calculate this number, then, as is shown in Figure 2, there is a correlation ($P < 0.01$) between this and the mean fruit weight for the variety, Granny Smith excepted.

This relation between characteristic varietal cell number and fruit weight is independent of rate of maturation and susceptibility to disorder.

(*b*) *Mean Cell Volume*

Again assuming that the data provide an approximate measure of the mean cell volume for the variety, then, as is shown in Figure 3, there is a group of varieties, Golden Delicious, Delicious, Crofton, Democrat, Sturmer, Cleopatra, Tasman Pride, and Jonathan, that does not conform to the relationship between cell size and maturation period established by Smith (1940) for English varieties. It is of interest that the varieties grown in both countries, Cox and Worcester, had similar cell size and growing period in each.

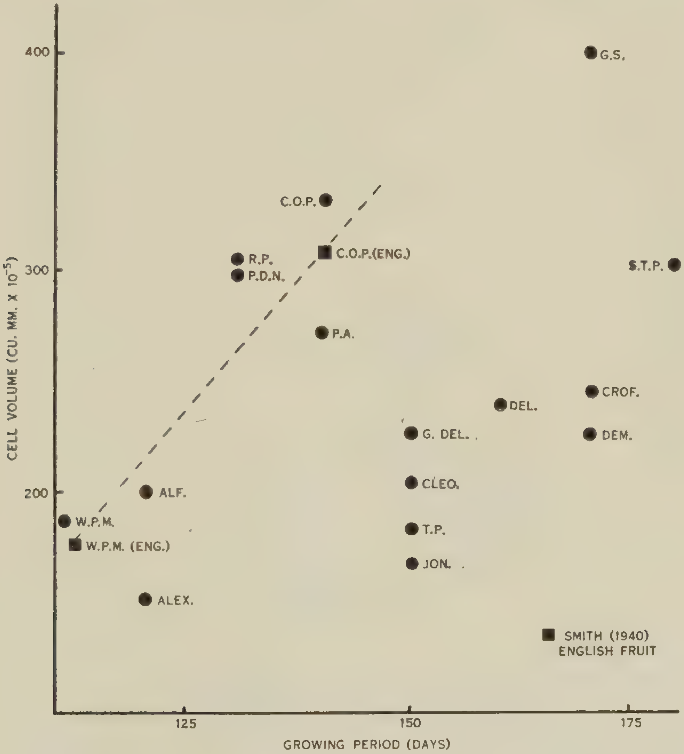

Fig. 3.—Relation between mean cell volume and growing period for different varieties.

(*c*) *Respiration Rate and Protein Nitrogen*

Most varieties can be assumed to be harvested in the preclimacteric stage. The respiration rate per cell was lower for pick 1 of Democrat than for pick 2, which would be expected from the work of Robertson and Turner (1951) and indicates that the latter picking was nearer the climacteric rise. The higher rate of the second picking of Granny Smith may have been due to the same effect. On the other hand, the lower value for pick 2 of Cox was probably due to the peak of the climacteric occurring between the pickings. In all

comparisons of varieties involving respiration, second pickings have been omitted. It is again assumed that the data provide an approximation to the varietal mean.

Respiration per cell is closely correlated with cell volume ($P < 0.01$) (Fig. 4), both between light and heavy crops and between varieties. This extends the scope of the relation reported within fruits of the one tree by Robertson and Turner (1951).

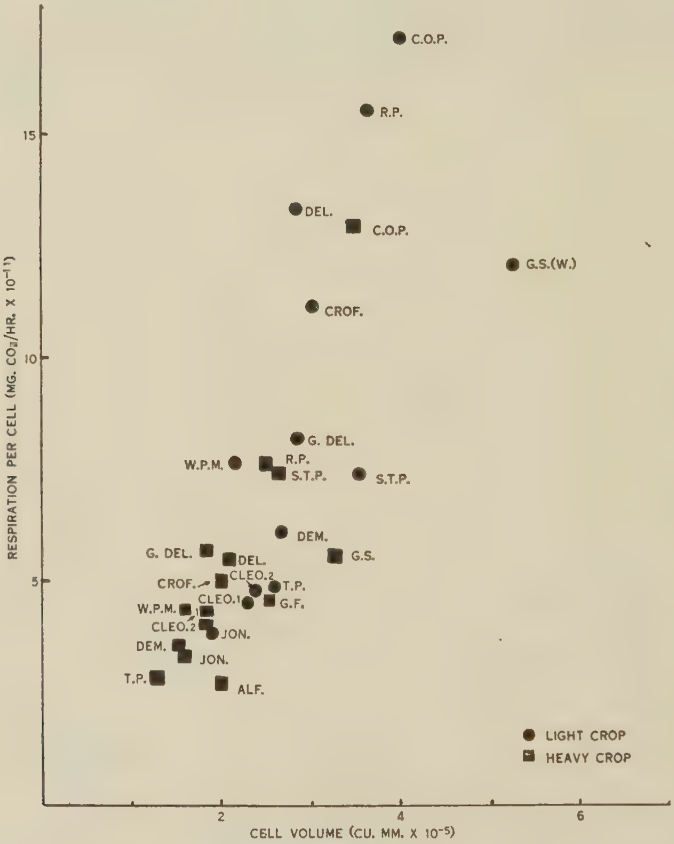

Fig. 4.—Relation between respiration rate per cell and cell volume for different varieties at two cropping levels.

Protein nitrogen per cell is also correlated with cell volume ($P < 0.01$) (Fig. 5) and cell surface ($P < 0.01$) (Fig. 6). This indicates that synthesis of protein keeps pace with increase in cell size. As the latter relation does not depart significantly from linearity, it is probable that the protoplasm is of constant thickness over this range of cell size, again extending the within-tree relation found by Robertson and Turner (1951).

If the data are examined on the basis of Hulme's (1951) conception of the respiration per unit protein, the general pattern is for light crop fruit to have a higher respiration per unit protein than heavy. In the only varieties where

this was not so (Tasman Pride and Golden Delicious) there was no significant difference.

There is a significant correlation ($P < 0.01$) between respiration per cell and protein nitrogen per cell but it appears that at higher respiration rates the relationship is not linear (Fig. 7). One possible interpretation of this is that,

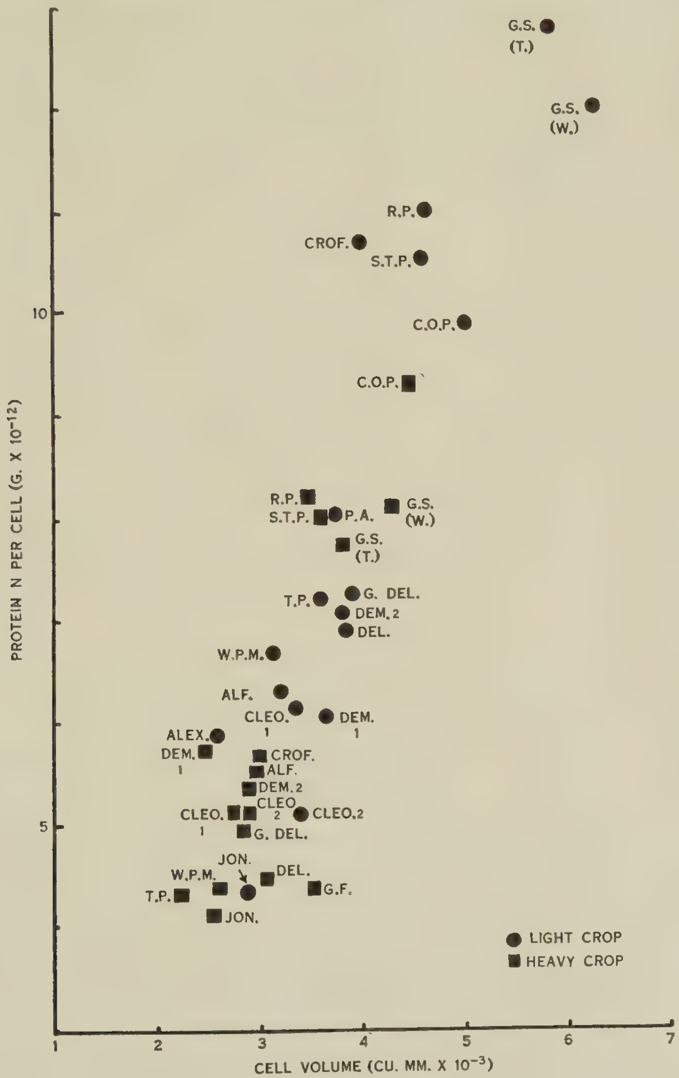

Fig. 5.—Relation between protein nitrogen per cell and cell volume for different varieties at two cropping levels.

with increasing protein content, the respiration necessary to maintain a given amount of protein increases also.

Excluding Granny Smith, correlations ($P < 0.01$) are found between respiration per unit protein and cell volume (Fig. 8) and between preclimacteric

respiration rate per unit fresh weight and number of cells per gram; the latter (Fig. 9) is negative in contrast with that found by Smith (1940) between post-climacteric respiration rate and number of cells per gram.

IV. Discussion

The more rapid senescence and greater susceptibility to disorder of light crop fruit may be related to its higher respiration per unit protein. Cells of light crop fruit appear to require more energy from respiration to maintain their protein. Possibly light crop cells are unable to maintain an efficient transfer of energy at the higher rates required and hence show more rapid senescence and greater susceptibility to disorder.

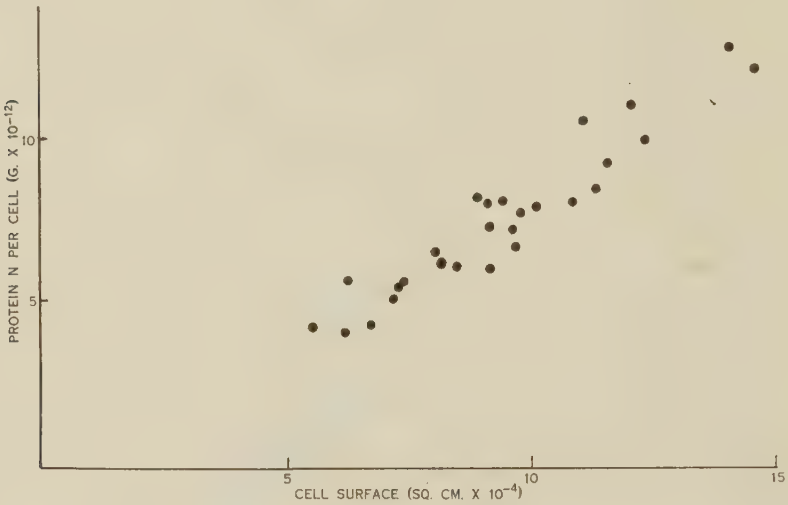

Fig. 6.—Relation between protein nitrogen per cell and cell surface for different varieties.

It is now possible to suggest an explanation why the larger fruit from heavy crops has a lower susceptibility to disorders and a slower rate of senescence than fruits of similar size from light crops, as reported by Carne and Martin (1938). In the former fruit, the large size has been shown by Bain and Robertson (1951) to be due mainly to increase in cell number and in the latter the larger size has now been shown to be due to increased cell size. Respiration per unit protein (R/P) would therefore be lower in the former.

The facts presented here support the theory of Robertson and Turner (1951) that the greater the protein concentration in an apple cell, the greater would be the transfer of energy from respiration to protein synthesis for maintenance. If the energy transfers took place through phosphate carriers, the more rapid dephosphorylation of carriers might result in an accelerated respiration since respiration rate depends on the amount of carrier available to accept phosphate. Robertson and Turner thought that large fruit might have difficulty in maintaining cell constituents where high protein contents were mak-

ing severe demands on the energy distributors of the cells. It has now been shown that though protein synthesis keeps pace with cell enlargement, the respiration per unit protein has increased with cell size.

The comparison of varieties, while of interest, has not shown any consistent correlation between cell characters such as cell size or cell number and physiological characters such as period required for maturation or date of maturation. Lack of any relation between R/P ratio and maturation period was unexpected.

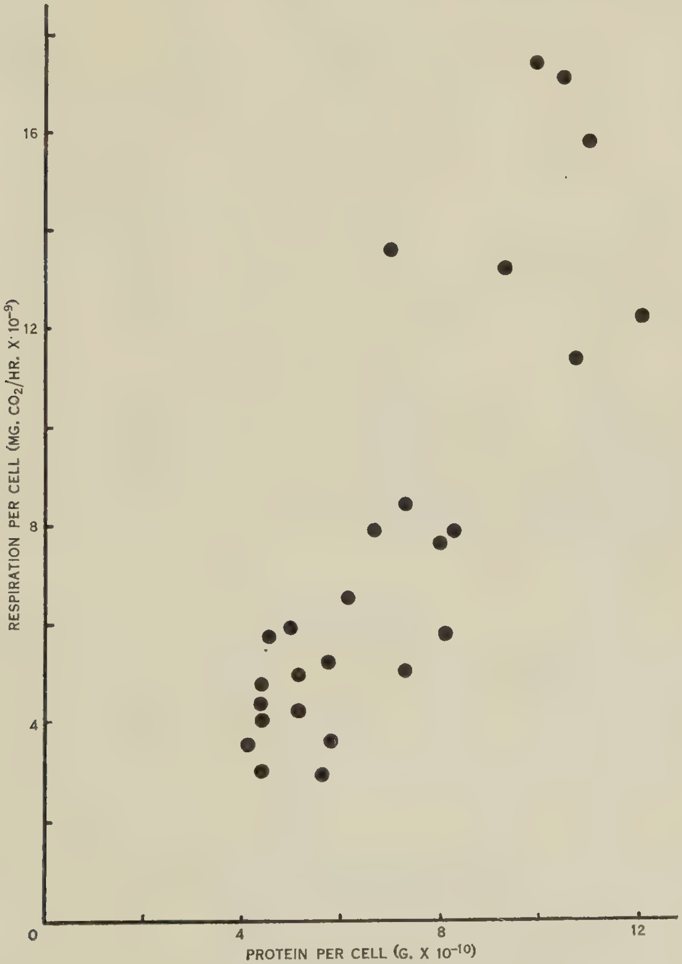

Fig. 7.—Relation between respiration rate per cell and protein nitrogen per cell.

There was a wider range in this value in Tasmanian dessert varieties than in English ones and though the only culinary variety studied (Alfriston) had a low value, it is felt that the transition from purely culinary to purely dessert quality is so gradual over the range of varieties that it is dangerous to assume that culinary quality is linked with R/P ratio. Sturmer, with a high R/P ratio,

has a high culinary quality; Tasman Pride with a low culinary quality a low one. This is the reverse of the relation found by Hulme (1951).

The anomalous behaviour of the variety Granny Smith (Figs. 2, 8, 9) is of interest but no explanation can be offered. Smith (1950) thought certain aberrant characteristics in Bramley's Seedling might be due to its triploidy, but Granny Smith is a diploid and Ribston, another triploid, appears to be normal.

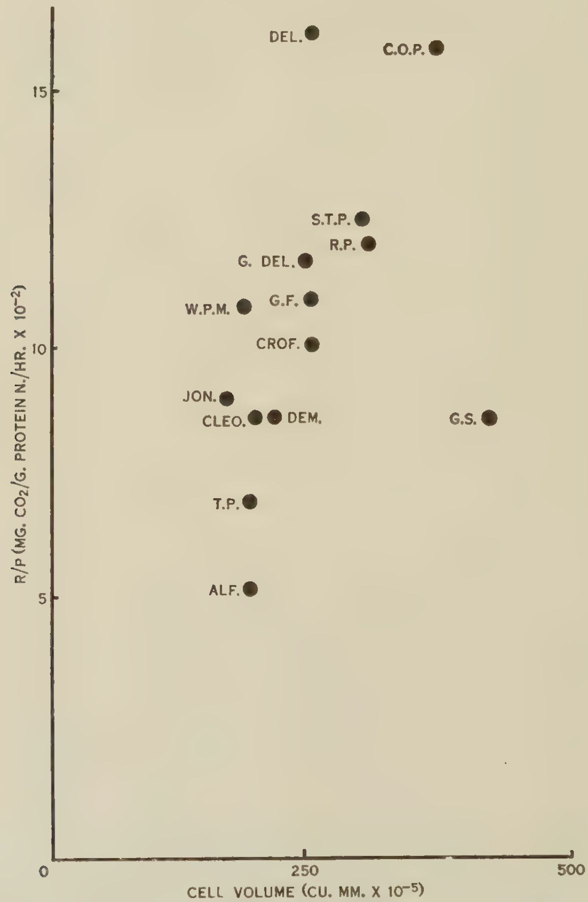

Fig. 8.—Relation between respiration per unit protein and cell volume.

One of the most important problems of the Tasmanian fruit industry is to increase the keeping quality of large fruit for which there is an increasingly strong demand. In the United States large fruit can be cool-stored for periods longer than is possible with Tasmanian fruits of the same varieties. Attempts to raise the mean fruit size without impairing keeping quality seem most likely to succeed if cell number per fruit is increased and cell size kept small.

Cell division normally ceases 3-4 weeks after pollination and therefore horticultural practices that stimulate cell division or prolong this period are likely to be beneficial while excessive increase in cell size must be avoided.

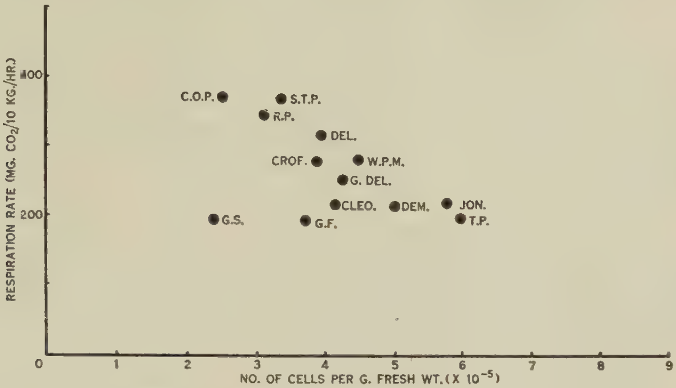

Fig. 9.—Relation between respiration rate per unit weight of tissue and number of cells.

V. Acknowledgments

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VOLATILE PRODUCTS OF APPLES

III. IDENTIFICATION OF ALDEHYDES AND KETONES

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Summary

The volatile aldehydes and ketones produced by whole Granny Smith apples at 30°C. were identified by paper chromatography and spectral absorption of the dinitrophenylhydrazones, and by conversion of the aldehydes to hydroxamic acids. Acetaldehyde was found to be the major constituent, with smaller amounts of propionaldehyde and acetone.

I. Introduction

A systematic study of the volatile substances produced by apples is being made in relation to the problem of superficial scald, a functional disorder of cold-stored fruit. A study of the volatile acids and alcohols has recently been published (Thompson 1951*b*). This paper is concerned with the identification of aldehydes and ketones.

Power and Chesnut (1920) obtained the volatile carbonyl compounds by passing air over whole apples and then through bisulphite solution. They identified acetaldehyde by the Rimini test.

White (1950) examined a volatile fraction of apple juice, and found acetaldehyde, acetone, caproaldehyde, and 2-hexenal. The dinitrophenylhydrazones were separated and identified by melting points and elementary analysis. One cannot be certain that all these substances are present in whole apples, as they may be produced during extraction and distillation of the juice.

This paper describes a further study of the volatile carbonyl compounds produced by whole apples. The methods of White were not applicable to the comparatively small quantity of material available, and use was made of paper chromatography and spectral absorption.

II. Collection of Sample from Air Stream

Air, purified by combustion of organic matter and absorption of carbon dioxide, was passed over 10 kg. of Granny Smith apples at 30°C. The apples had been picked commercially mature and subsequently stored for 4-7 months at 0°C. The volatile carbonyl compounds were absorbed in 30 ml. of concentrated metabisulphite solution (25 g. $K_2S_2O_5$ per 100 ml.) contained in a spiral absorber cooled in ice. After 4 days the absorbing solution was transferred to a stoppered bottle, and the absorber was washed with a small amount of water.

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After each collection the apples were examined for rots and other lesions. In the course of 18 collections only one rot, 1 cm. in diameter, and no other lesions were found. Hence the substances identified were definitely produced by sound tissue.

The absorbing solutions and washings from several collections were bulked in the bottle and stored at 1°C. This bulk solution was finally made alkaline with sodium carbonate (to liberate the free carbonyl compounds) and distilled to about one-quarter of the original volume. The distillate was collected in an ice-cooled receiver and stored at 1°C. It was free from volatile acids but contained alcohols and a trace of esters in addition to carbonyl compounds.

III. Conversion to Dinitrophenylhydrazones

A solution of the dinitrophenylhydrazones of the carbonyl compounds was prepared as follows. To 40 ml. of the aqueous distillate was added 25 ml. of 2,4-dinitrophenylhydrazine solution (0.1 per cent. in 1N HCl), and the mixture was allowed to stand for 1 hour at 25°C. Longer periods of standing up to 24 hours made no difference to the final result. The dinitrophenylhydrazones were extracted with 25 ml. of a mixture of light petroleum and benzene in equal proportions. The solution of dinitrophenylhydrazones was washed with small amounts of water, filtered, and evaporated to about 20 ml. It was then washed into a 25 ml. volumetric flask and made to volume with light petroleum.

The volatile ketones were converted separately to dinitrophenylhydrazones after removal of the aldehydes by oxidation. The most suitable oxidizing agent was found to be freshly precipitated silver oxide in an alkaline solution. This oxidized dilute solutions of aldehydes very readily with little effect on ketones or alcohols. In the procedure adopted, 40 ml. of the aqueous distillate was mixed with 5 ml. of 1N silver nitrate followed by 5 ml. of 2N sodium hydroxide. All solutions were cooled to 0°C. before mixing. The mixed solution was shaken for 1 hour at 0°C. The precipitated silver oxide was then filtered and washed at 0°C. The filtrate was acidified with 5 ml. of 2N hydrochloric acid. The derivatives of the ketones were prepared from the filtrate by the procedure already described, and the solution of the dinitrophenylhydrazones was made to 25 ml.

IV. Paper Chromatography of Dinitrophenylhydrazones

The separation of the dinitrophenylhydrazones of simple aldehydes and ketones by paper chromatography was investigated in order to identify the volatile products of the apple. The system water-butanol, recommended by Cavallini, Frontali, and Toschi (1949) for the derivatives of keto acids, was found unsuitable for simple aldehydes and ketones, whose derivatives travelled practically with the front. Workers of the Food Investigation Board of Great Britain have proposed the system methanol-heptane, and this system was found suitable for separating simple aldehydes and ketones containing up to four

carbon atoms. A petroleum fraction boiling between 95 and 105°C. was used in place of pure heptane.

The procedure involved upward movement of solvent in a relatively simple apparatus. A battery jar, 8 in. square and 20 in. high, contained a flat, circular dish of diameter 6 in. Spots containing approximately 10^{-7} mole of each derivative were placed about 1.5 in. from the bottom of a sheet of Whatman No. 1 paper 18 in. high. Spots should be not less than 1.5 in. apart. The sheet was formed into a cylinder and placed in the inner dish.

Methanol (50 ml.) and "heptane" (100 ml.) were shaken together in a separating funnel. The mixture separated into approximately 75 ml. of each phase. The lower, methanol-rich phase was transferred to the bottom of the battery jar outside the dish carrying the paper. The jar was covered by a glass plate sealed with grease and the system was left to equilibrate overnight. A square of filter paper pasted to the bottom of the glass plate was found to assist equilibration and prevent any condensate dripping back. In the morning the "heptane" phase was transferred to the inner dish and the glass cover replaced. The mobile phase travelled fairly rapidly up the paper, which was usually removed after 6-8 hours.

On drying the paper in a current of air the dinitrophenylhydrazones were visible as faint yellow spots. Spraying with 10 per cent. aqueous sodium hydroxide gave much more intense colours — dark orange with simple aldehydes and ketones, and red to blue with some dicarbonyl compounds.

R_F values of the same derivative varied somewhat in different runs, but were in good agreement when taken from the same paper. Hence derivatives from the apple could be readily identified by running them alongside known derivatives in the same chromatogram. The usual values for simple aldehydes and ketones were as follows: formaldehyde 0.35-0.40, acetaldehyde 0.50-0.55, propionaldehyde and acetone 0.70-0.75, butyraldehyde and methyl ethyl ketone 0.85-0.90. The R_F values of the aldehyde and ketone of the same carbon number did not differ significantly. Diacetyl travelled level with formaldehyde and furfural just behind, while glyoxal remained at the origin.

The derivatives from the apple were identified by the following procedure. Spots containing derivatives of (*a*) aldehydes with one to four carbon atoms, (*b*) apple aldehydes and ketones, (*c*) apple ketones, and (*d*) ketones with three and four carbon atoms were placed from left to right on the same paper. Equivalent amounts of the apple derivatives (0.1 ml. of each solution) were contained in spots *b* and *c*. A photograph of the chromatogram (using panchromatic film with a green filter) is shown in Plate 1.

Among the apple derivatives acetaldehyde predominated with smaller amounts of propionaldehyde and acetone. The presence of both propionaldehyde and acetone was indicated by the fact that the spot containing both derivatives (3*b*) was larger than the spot containing acetone alone (3*c*). This conclusion was confirmed by tests to be described in subsequent sections. Faint spots, which gave a similar orange colour on spraying, remained at the origin. The spots at the origin were not obtained if the solution of the derivatives was first poured through a column of anhydrous magnesium sulphate.

V. Spectral Absorption of Dinitrophenylhydrazones

Solutions of the dinitrophenylhydrazones in ethanol gave characteristic colours on addition of ethanolic potassium hydroxide. The colour faded slightly after mixing but became relatively stable after the first hour. An aliquot of a dilute ethanolic solution of the derivative was measured into a 10 ml. volumetric flask, followed by 1 ml. of 0.5N ethanolic potassium hydroxide and sufficient ethanol to bring to volume. After standing for an hour the absorption was measured between 400 and 700 mμ with a Beckman DU spectrophotometer.

The derivatives of the simple aldehydes and ketones all gave a maximum absorption at 420-430 mμ, and the absorption curves did not differ sufficiently to distinguish readily between aldehydes or ketones of different carbon number. There was, however, a slight but characteristic difference between the aldehyde and ketone curves, which was used to confirm the presence of acetone in the apple volatiles. The absorption of the ketone derivatives gave a slight rise from 490 to 520 mμ with a subsidiary maximum at 520 mμ, while the aldehyde curves only gave inflections in this region.

A solution of acetone derivative from the apple was obtained by the following procedure. The solution of apple volatiles (200 ml.) was oxidized with silver oxide and the derivatives prepared. The solution of dinitrophenylhydrazones in equal parts of light petroleum and benzene was poured through a column of anhydrous magnesium sulphate and washed through with further solvent mixture. The solution was evaporated to dryness and the residue crystallized twice from light petroleum. The purified derivative was dissolved in benzene and applied as a series of spots on sheets of filter paper. Chromatograms were run, and the spots corresponding to acetone were cut out and extracted with ether. The ether was evaporated and the derivative made to 10 ml. with ethanol. An aliquot of this solution was used for obtaining the spectral absorption curve.

For comparison of the apple derivative with known derivatives, the function $\log D_\lambda - \log D_{400}$ was plotted against wavelength (D_λ = optical density at wavelength λ and D_{400} = density at 400 mμ). This function is independent of concentration where Beer's law is obeyed, i.e. up to 2×10^{-4}M for the dinitrophenylhydrazones concerned. The curves of the derivatives of pure propionaldehyde and acetone, and of the apple derivative, are given in Figure 1. The curve of the apple derivative corresponds closely to that of the acetone, and is different from that of the propionaldehyde derivative.

The concentration of mixed aldehyde and ketone derivatives from the apple was calculated approximately from the absorption at 420 mμ, taking acetaldehyde as standard. The production of total carbonyl compounds by the apples was estimated at about 7 μmoles/kg./24 hr. This figure is only approximate, as the method has not been adapted for quantitative work.

VI. Conversion of Aldehydes to Hydroxamic Acids

The presence of acetaldehyde and propionaldehyde was confirmed by conversion to hydroxamic acids which were identified by paper chromatography

(Thompson 1951*a*). The solution of apple volatiles (40 ml.) was oxidized with silver oxide as previously described. The filtrate was acidified with sulphuric instead of hydrochloric acid. The oxidized solution was then distilled to about one-fifth of the original volume. The distillate, which contained the acids from oxidation of aldehydes, was neutralized to pH 8 with dilute sodium

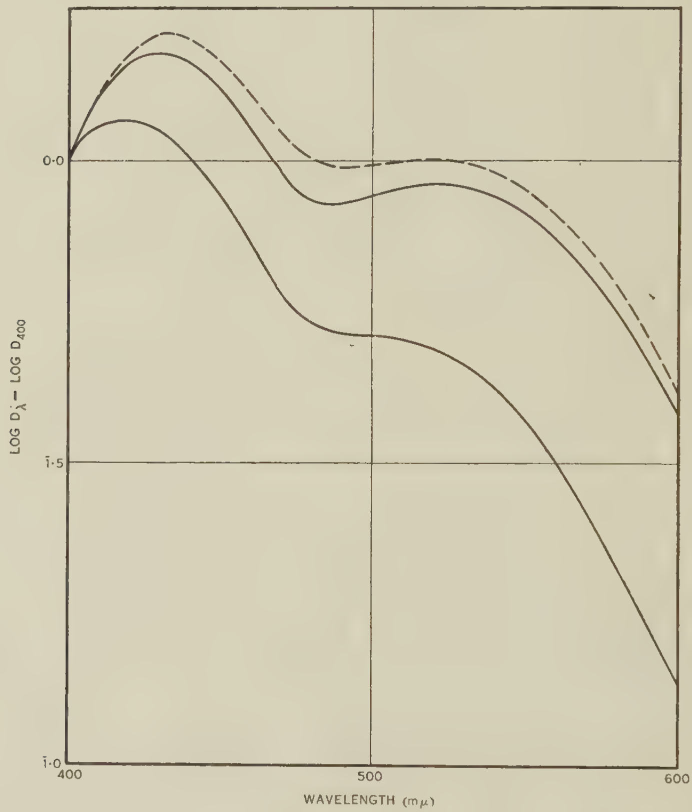

Fig. 1.—Spectral curves of dinitrophenylhydrazones. Propionaldehyde (pure) : lower continuous curve. Acetone (pure) : upper continuous curve. Acetone (apple) : broken curve.

hydroxide and evaporated to dryness. The dry residue of sodium salts was converted to hydroxamic acids by the method of Thompson (1951*b*), and half the total quantity was applied as a spot to the paper. In a chromatogram using amyl alcohol and acetic acid, hydroxamic acids containing one to four carbon atoms were run alongside hydroxamic acids derived from the apple aldehydes. A photograph of the chromatogram in shown in Plate 2.

The presence of acethydroxamic and propionhydroxamic acids is clearly shown, confirming the presence of acetaldehyde and propionaldehyde in the original solution of volatiles. There was no interference by free acids in the original solution, as these were in negligible concentration. To investigate

interference by esters, a further 40 ml. of the original solution was extracted with ether and the esters converted to hydroxamic acids. Half the total quantity (equivalent to the hydroxamic acids from aldehydes) was applied to the paper. The chromatogram showed a comparatively faint spot in position 2 and none in position 3, indicating negligible interference.

As silver nitrate was used in the oxidation of the aldehydes, the distillate and the sodium salts obtained on evaporation may have contained nitrate. It was demonstrated that 30 mg. of sodium nitrate gave no spots on the chromatogram. As a further check, the oxidation was repeated with an equivalent amount of silver sulphate (instead of nitrate) but with no other modification. On account of its low solubility solid silver sulphate was added directly to the solution of volatiles and shaken for 30 minutes before adding sodium hydroxide. As before only acethydroxamic and propionhydroxamic acids were obtained on the chromatogram.

Another sample of sodium salts, free from nitrate, was tested for unsaturation by measuring hydrogen uptake in the presence of colloidal palladium (Milton and Waters 1949). The result indicated less than one double bond in 100 moles of aldehydes.

VII. Discussion

Acetaldehyde and propionaldehyde have now been identified as the predominant volatile aldehydes produced by whole Granny Smith apples at 30°C. after 4-7 months at 0°C. In contrast White (1950) found acetaldehyde, caproaldehyde, and 2-hexenal in the volatile portion of juice derived from McIntosh and Stayman Winesap apples. It is plausible to assume that intact tissue produces caproaldehyde and 2-hexenal as well as the lower aldehydes. This assumption is supported by the identification of hexyl alcohol and caproic acid as products of whole apples (Thompson 1951*b*). On this view the difference in the aldehydes identified would be due to variety or the physiological state of the fruit rather than to changes during the preparation and distillation of the juice. It is even possible that other aldehydes may predominate in the volatile products of Granny Smith apples at lower temperatures.

Fatty acids and alcohols containing one, two, three, four, five, and six carbon atoms have been identified in the volatile products of apples (Thompson 1951*b*; White 1950). The aldehydes may be intermediates in the interconversion of acids and alcohols, and acetaldehyde probably arises also from decarboxylation of pyruvic acid, an important intermediate of respiration.

The acetone is probably a product of fatty acid metabolism. It could arise from decarboxylation of aceto-acetic acid, an intermediate in the β-oxidation of fatty acids.

VIII. Acknowledgment

The author is indebted to Mr. Bruce Kennett for technical assistance.

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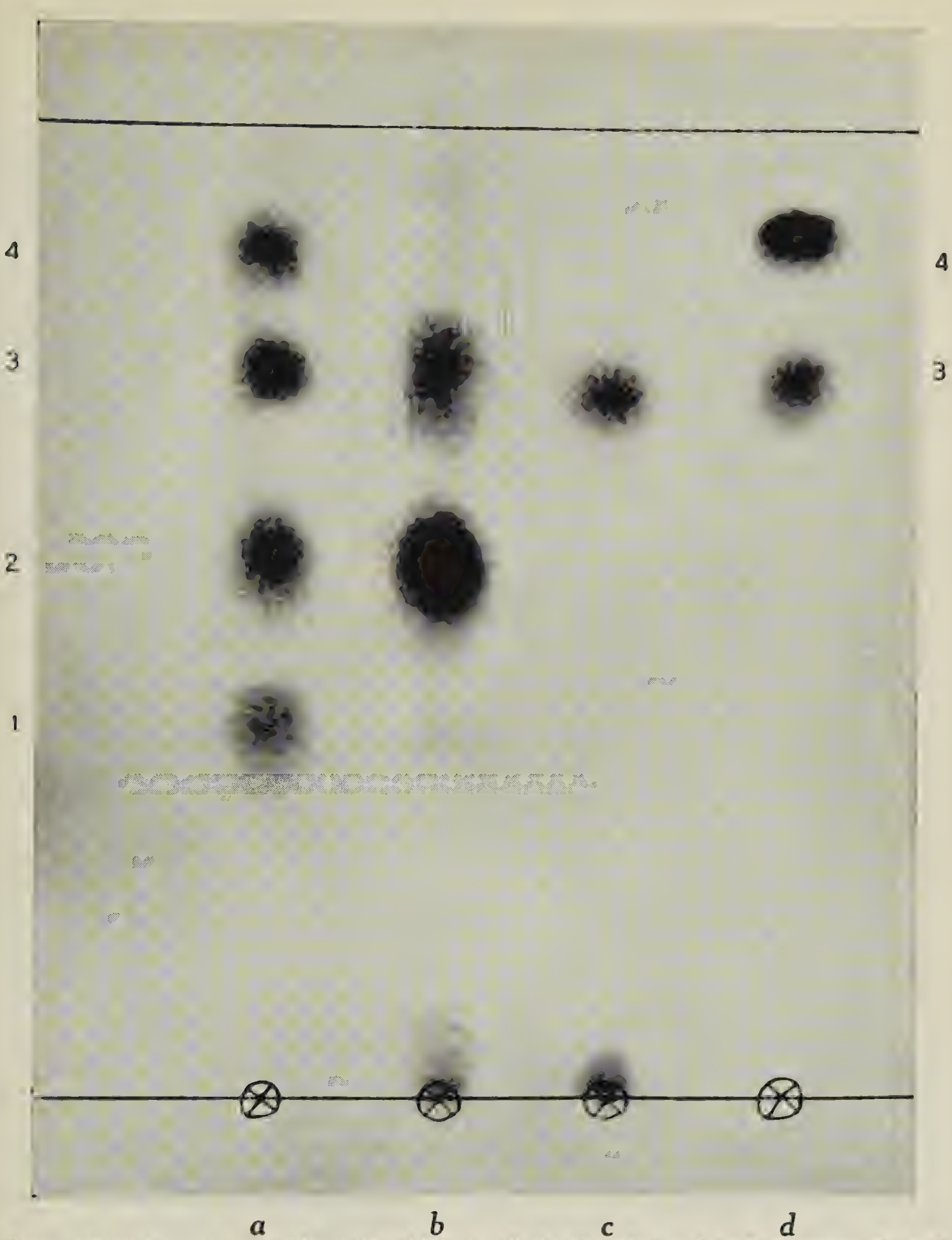
VOLATILE PRODUCTS OF APPLES. III



Paper chromatogram run with methanol-"heptane" showing dinitrophenylhydrazones derived from:

(*a*) Aldehydes with one to four carbon atoms (as numbered),
(*b*) Apple aldehydes and ketones,
(*c*) Apple ketones, and
(*d*) Ketones with three and four carbon atoms.

Paper chromatogram run with amyl alcohol-acetic acid showing ferric hydroxamate spots derived from:

(*a*) Hydroxamic acids with one to four carbon atoms (as numbered),
(*b*) Apple aldehydes, and
(*c*) Propionhydroxamic acid.

ELECTRIC POTENTIAL DIFFERENCES IN BEAN ROOTS AND THEIR RELATION TO SALT UPTAKE

By A. B. Hope* and P. G. Stevens†

[*Manuscript received January* 31, 1952]

Summary

Reversible diffusion of KCl is shown to occur between an aqueous solution and young bean roots, probably in the protoplasmic phase. Evidence for this is presented from a study of electric potential difference (p.d.) changes and changes in environmental salt concentration in different samples but under comparable conditions.

Characteristic changes of p.d. with time are obtained when a root tip taken from distilled water is dipped in 0.01N KCl followed by 0.0001N KCl, indicating an increase of concentration in the root tissue while in the first solution and then a decrease while in the more dilute solution.

When excised root tips which have been in distilled water are immersed in 0.01N KCl, conductivity measurements show a mean initial uptake of 0.13×10^{-5} g. mol./g. of tissue. An approximately equal amount of salt leaves the roots if they are then transferred to 0.0001N KCl.

The region of the tissue to which the electrolyte has access by diffusion, called the "apparent free space," is calculated to be 13 per cent. for the material used. Approximately 10 of the 13 per cent. is likely to be protoplasm.

The evidence supports the hypothesis that electric p.d.'s between young root tissue and surrounding electrolytes are the result of differential mobility of cations and anions in the protoplasmic phase of the epidermal cells.

The relation of the evidence to theories postulating a high resistance barrier to ion diffusion at the boundary of protoplasm and environment is discussed. It is concluded that the properties of the protoplasm/environment boundary do not include high resistance to ion diffusion. It is possible that protoplasm contains a certain concentration of non-mobile anions resulting in a Donnan distribution of ions between this phase and the surrounding solution. Ways of testing this hypothesis are suggested.

I. Introduction

In an earlier paper (Hope 1951) the results were given of investigations concerning electric potential differences (p.d.) appearing between solutions of electrolytes of various concentrations applied at two loci on broad bean roots. It was shown that these could be interpreted as diffusion p.d.'s between the root phase and the surrounding solution. These p.d.'s were caused by differences of concentration of electrolyte and in mobility of anion and cation in

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diffusion between the environment and what was presumed to be the protoplasmic phase of the root cells. These results were compared with those of Lundegardh (1940) and agreement found in most instances but it was pointed out that Lundegardh's results, which he concluded were consistent with an adsorption of ions to a lipoid-type membrane at the boundary between protoplasm and environment, could equally well be interpreted on the basis of diffusion of ions.

In Section III (*e*) of that paper (Hope 1951) experiments were reported in which potential changes with time seemed to show a reversible change of concentration of the root phase corresponding to changes in concentration in the environment. These experiments have been repeated under more controlled conditions to eliminate spurious p.d. changes at the reference point on the root. Also, evidence is presented from a study of the conductivity changes in solutions containing root tips to show that there is movement of salt into the root phase from a concentrated solution by means of diffusion. This is followed by back leakage of the salt if the root is transferred to a more dilute solution. An estimate is made of the percentage of the root tissue concerned with the free diffusion of salt. The nature of the phases into which salt can diffuse reversibly is discussed. Experiments are suggested with which to test the hypothesis that the root protoplasm contains a bulk distribution of immobile anions.

II. Experimental Method

Broad bean (*Vicia faba* L.) roots were grown as described in the earlier paper. In the present experiments, however, instead of being taken immediately from the moist spaghnum (of unknown ionic composition) the roots when 2-3 cm. long were held in a perspex trough containing aerated distilled water and equilibrated for 17-24 hr. at a fairly constant temperature of $18 \pm 3°C$. In the experiments concerning the uptake of salt by root tissue, a number of tips 5 mm. long (containing the meristematic region and elongating cells) were excised from roots between 2 and 4 cm. long and, with several changes of water, equilibrated for 17-24 hr. in a flask aerated with moist air.

(*a*) *P.D. Experiments*

Reference should be made to Hope (1951) for details of nomenclature and technique. However, the following modifications were made. Tenth-normal calomel half-cells dipping into glass tubes containing 0.1N KCl and 10 g. agar/l. were used to make contact with the various solutions surrounding the root. The p.d.'s were measured with a high input resistance valve electrometer.

The roots were removed from the distilled water and the surface mopped dry of excess moisture. Contact at the *b* region was established 20-25 min. before the time zero of Figure 1. This was done by lowering the root, held at an angle of 20-30° from the horizontal, onto a polystyrene cup holding 0.0001N KCl into which dipped the reference contact tip. If this contact was restricted to a small area of the root, liquid did not tend to travel to the *a* region and the two loci were kept insulated from each other.

(b) *Conductivity Experiments*

The method of following salt uptake by measuring the change in conductivity in the electrolyte surrounding the living tissue is substantially the same as that used by Robertson (1941). The tissue : solution ratio was usually 0.7 g. root tips: 10 ml. solution. No significant difference in salt uptake was found between whole and excised root tips, so the cut tips were adopted because of convenience since the tips of whole roots with their large seeds are difficult to arrange in a small volume of solution. In addition, with whole roots a variable volume of tissue (greater than that immersed in the solution) is concerned with absorption and the uptake per gram of tissue is difficult to measure. The excised tips were transferred from distilled water to a small cup containing 10 ml. of KCl, which was aerated gently with moist air, the whole being held in a perspex box through which moist air was flowing. In each experiment the temperature was constant within $\pm 1°C$. Samples of *c.* 0.5 ml. of solution were withdrawn for measurement of conductivity at intervals. Each of these was returned to the experiment just before the next sampling.

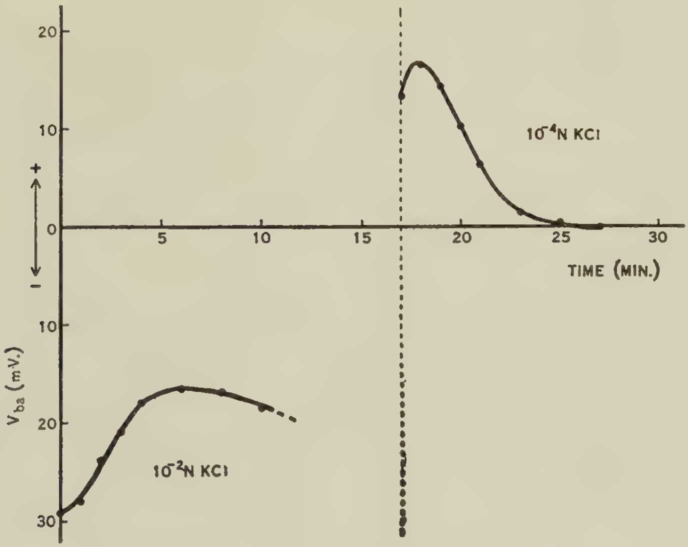

Fig. 1.—Variation of p.d. at the root — solution interface at *a* (tip of root) with time. The roots were immersed first in 0.01N then 0.0001N KCl. The sign of the p.d. is that of the solution at *a* relative to the reference solution at *b* (10-20 mm. from tip). The points plotted are the means for five experiments.

III. Results

(a) *Change with Time of the Potential Difference between Root Tips and 0.01N, 0.0001N KCl Solutions*

Figure 1 shows that the potential of a 0.01N KCl solution applied to the region "*a*" at the tip of the root becomes more positive by up to 12 mV. in the

first 5 min. with respect to a reference solution at "*b*" some 10-20 mm. from the *a* region. If the 0.01N solution is then removed and 0.0001N KCl substituted, the characteristic quick change in p.d. is obtained, the solution at *a* immediately becoming some 40 mV. more positive but then drifting *c.* 15 mV. more negative in the following 5 min.

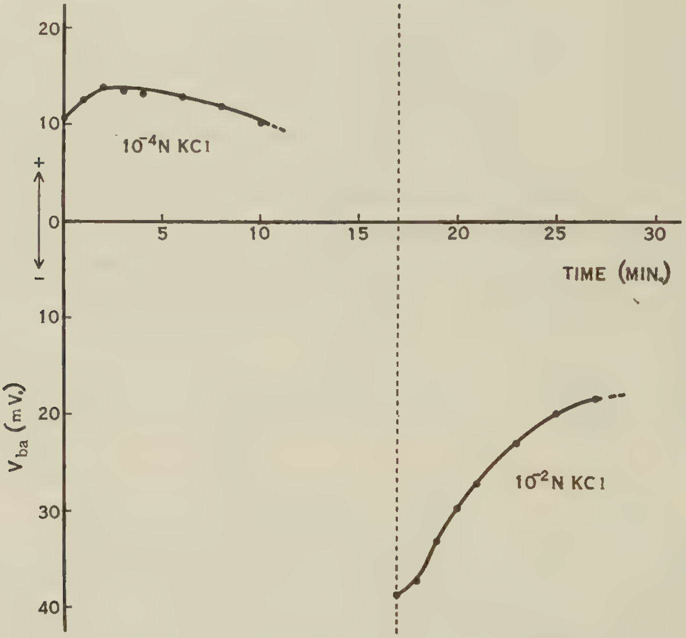

Fig. 2.—Variation of p.d. with time when the root tips are immersed in 0.0001N, then 0.01N KCl. The sign convention is as for Figure 1. Mean of five experiments.

It has previously been shown that for quick changes in salt concentration, the potential difference between the reference point *b* and the solution surrounding *a* could be represented approximately by the equation for diffusion potentials:

$$V_{ba} = k + 58 \frac{u - v}{u + v} \log_{10} \frac{c_2}{c_1} \quad \ldots \quad \ldots \quad (1)$$

where the p.d. is in mV. at 20°C. and the sign that of *a* relative to *b*; *u, v* are the mobilities of the cation and anion in the phase in which they are diffusing; c_2 the inside and c_1 the outside salt concentrations; and *k* a constant. In previous experiments approximately 30 mV. change per tenfold change in [KCl] was obtained, and the ratio *u/v* was calculated to be approximately 3. In later experiments the change varied between 20 and 25 mV./tenfold concentration change. If V_{ba} changes with time for constant c_1 then *k*, *u/v*, or c_2 must be a function of time. *k* includes the phase p.d. between the solution at *b* and the root and this must be assumed nearly constant in the time intervals involved

since the contact is made well before the circuit is completed by dipping the tip into solution. This was not the case in the experiments reported earlier in which the reference point at b was variable owing to contact being made with 0.1N KCl only some 5-10 min. before that at a. Also, u/v must be nearly constant since normal quick changes in p.d. occur with quick changes of c_1, viz. 20-25 mV./tenfold concentration change. Thus the results seem to suggest that c_2 increases as the tip remains in 0.01N KCl and decreases again when in 0.0001N KCl.

Figure 2 shows that if the root tip is taken from distilled water and the first contact at a made with 0.0001N KCl then V_{ba} remains approximately constant for 10 min. The application of 0.01N KCl then gives a negative change of 40-50 mV. in V_{ba} followed by a positive drift of nearly 20 mV. This is consistent with the diffusion hypothesis since the root phase in which diffusion can occur is presumably free of diffusable ions after being treated with distilled water. Thus an initial application of 0.0001N KCl causes much less change in c_2 than the subsequent application of 0.01N KCl.

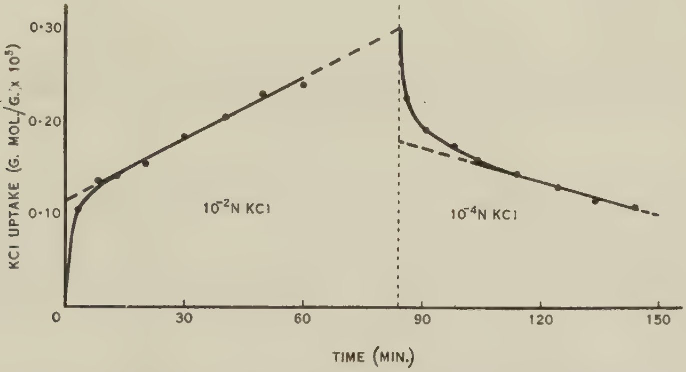

Fig. 3.—Initial uptake and accumulation followed by leakage of KCl by excised root tips immersed in 0.01N and 0.0001N KCl solutions respectively.

(*b*) *Uptake of KCl by Excised Root Tips*

The conductivity method (Robertson 1941) was used to find the changes in concentration that occur in KCl solutions of various concentrations, surrounding root tips. It is shown that when tips of broad bean roots are transferred from distilled water, the surfaces being carefully mopped dry of free liquid, to 0.01N KCl solution, the conductivity of the KCl decreases in a regular and repeatable manner. Figure 3 shows the result of a typical experiment. When in 0.01N KCl, the uptake of salt appears in two stages, an initial uptake (Robertson 1944; Robertson, Turner, and Wilkins 1947) in which usually $0.13 \pm 0.02 \times 10^{-5}$ g.mol./g. tissue leaves the solution in about 20 min., followed by a steady decrease in solution concentration at the rate of $0.14 \pm 0.04 \times 10^{-5}$ g.mol./g./hr. The latter is active accumulation by the respiratory mechanism

(Robertson 1941). If the tips are then transferred to 0.0001N KCl they leak an amount approximately equal to the initial uptake in 0.01N KCl and in a similar time, followed by a slow, steady leakage at the rate of $0.05 \pm 0.02 \times 10^{-5}$ g.mol./g./hr. (Fig. 3).

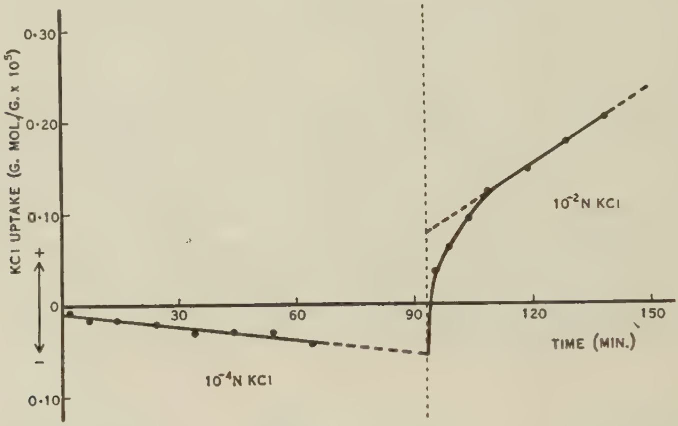

Fig. 4.—Slow leakage followed by initial uptake and accumulation of KCl by excised root tips immersed in 0.0001N and 0.01N KCl respectively.

Figure 4 gives a typical result of placing the equilibrated root tips immediately into 0.0001N KCl for approximately 1 hr. followed by treatment with 0.01N KCl. No marked initial leakage is observed in the dilute solution but an uptake of 0.12×10^{-5} g.mol./g. occurs in the more concentrated one, in about 20 min., followed by a steady uptake at the usual rate. These results should be compared with those obtained with excised sections of barley roots by Milthorpe and Robertson (1948). The accumulation rate for bean root tips is of the same order as that for the barley roots (0.45×10^{-5} g. mol./g./hr.) but the initial uptake in the barley experiments was approximately 1×10^{-5} g. mol./g. compared with $0.13 \pm 0.02 \times 10^{-5}$ g. mol./g. (an average of 15 replicates) for broad bean roots. This disagreement could be due to a difference in pH (see later) or to insufficiently dried roots in the barley experiments.

(*c*) *Distribution of Free Spaces in Bean Root Tissue*

The experiments just described show that there is a reversible diffusion of KCl into bean root tips of average amount 0.13×10^{-5} g. mol./g. when the tips are placed in a 0.01N solution. If that part of the tissue to which the KCl has access is empty of electrolyte to start with, then during the initial uptake 0.13 g. of each g. of tissue reaches a concentration of 0.01N. This proportion has been termed the "apparent free space"* (A.F.S.). The A.F.S. for the root

* This term has been used by G. E. Briggs (unpublished data) to describe the portion of the plant tissue into which substances in solution apparently move by free diffusion.

tissue is thus 13 per cent. It is possible that the KCl can diffuse reversibly into:

(i) Intercellular spaces which are injected with distilled H_2O;

(ii) Spaces in the cellulose wall lattice which likewise contain water with very little dissolved substance in it; and

(iii) The protoplasm of the cells.

Since the salt accumulated by the salt respiration mechanism does not leak out to any extent in very dilute solutions (Robertson and Thorn 1945) it is almost certain that the cell vacuoles are not concerned in the diffusion of KCl in the initial uptake and initial leakage.

(i) The mean intercellular space in the 5 mm. tip of bean roots has been estimated as follows. Transverse microtome sections, fixed and stained with haematoxylin, were placed in a microprojector and the cell outlines drawn on squared paper. The intercellular space was calculated from the difference between total area and area occupied by cells. The root cap, epidermal, and stele cells are closely packed but the cortical cells are more nearly circular in section. From 10 sections cut at various distances from the root cap, the mean intercellular space is 7 per cent. by area, and therefore 7 per cent. by volume. This may be an over-estimation if shrinkage occurred during fixation and staining but a living section, cut free-hand and non-stained, also had 7 per cent. intercellular space. However, nearly all the spaces between cells in the cortex are filled with air and not liquid, as shown in Plate 1, Figure 1, which is a photomicrograph of a transverse section hand-cut and placed in water. The darker spaces around the cells are evidently air bubbles. This air disappears if the section is placed in a vacuum for a short time, as seen in Plate 1, Figure 2. The contribution of the intercellular space to the A.F.S. is therefore small and may be zero if all spaces are filled with air.

(ii) It is only possible to make a rough estimation of the contribution by the cellulose wall spaces to the A.F.S. Wardrop* (personal communication) has suggested the following approach. The dry weight of the 5 mm. tips of broad bean roots is about 12 per cent. of the fresh weight. Brown and Sutcliffe (1950) suggest that about 25 per cent. of the dry weight of comparable sections of barley roots is cellulose. If this figure is applicable to bean roots then cellulose comprises about 3 per cent. by weight of the fresh tissue and less by volume.

If the cellulose network is associated with its own volume of water (a possibility suggested by Robertson†, personal communication) the cellulose walls contribute less than 3 per cent. to the apparent free space.

(iii) It is thought likely that up to 10 per cent. of the tissue, not including intercellular and cell wall spaces, is permeable to KCl. It is almost certain

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† Division of Food Preservation and Transport, C.S.I.R.O.

that this free space is protoplasm. It is hoped to be able to make an estimate of the amount of protoplasm in a given piece of bean tissue by a staining technique.

IV. Discussion

In experiments reported earlier (Hope 1951) it was strongly indicated that potential differences appearing in broad bean roots as a result of a difference of concentration of electrolyte applied at two insulated loci were due to diffusion p.d.'s set up at the boundaries between the root and solution.

The results given in Section III(*a*) of this paper, and summarized in Figures 1 and 2, support this hypothesis. They have been interpreted to show that the root phase increases in ionic concentration when transferred from distilled water to 0.01N KCl since the surrounding solution drifts positive with respect to a constant reference electrode. The subsequent negative drift when the root is transferred to 0.0001N KCl similarly indicates a decrease in root concentration. Since a 20-25 mV. change in p.d. has been shown to correspond to a tenfold change in outside concentration, the 10-20 mV. drifts in Figures 1 and 2 may be tentatively taken to indicate a 3-10-fold concentration change somewhere in the root.

These changes of concentration have been proved directly by following the changes in conductivity of solutions of KCl surrounding excised root tips. The graphs of Figures 3 and 4 show that about 0.13×10^{-5} g. mol. KCl/g. of root tissue enter the root tips in about 20 min. when they are taken from distilled water and dipped in 0.01N KCl. The same amount of KCl leaves the tips following a transfer from 0.01N KCl to 0.0001N KCl. The A.F.S., or that part of the tissue to which solute has access by diffusion, is calculated to be 13 per cent. by weight (and 13 per cent. also by volume). Consideration of the intercellular spaces indicates that these are not part of the A.F.S. since they are filled with air and, apart from a small contribution by spaces in the cellulose fibres of the cell walls, the A.F.S. must be largely protoplasm.

The ability of ions of an electrolyte to penetrate the protoplasm to more than a few molecular layers is contrary to Lundegardh's (1940) hypothesis that a lipoid-type membrane, containing fixed negative valencies and having a high resistance to ion movement through it, is present at the interface between protoplasm and surrounding medium. According to Lundegardh's ideas the first step in the active accumulation process was a passive adsorption of metallic cations to the membrane in exchange for hydrogen ions. In the light of the present evidence, and contrary to Lundegardh's theory, it seems more likely that ions which are later transported by the accumulation mechanism are able to diffuse without great difficulty into the bulk of the protoplasm. Whatever the nature of the interface between the protoplasm and the environment, its properties do not seem to include a high resistance to diffusion of the ions of KCl.

There have been several suggestions made that protoplasm contains a bulk distribution of negative immobile ions (as opposed to the Lundegardh monolayer; see particularly Blinks 1940; Briggs and Robertson 1948). The facts

BEAN ROOT POTENTIAL DIFFERENCES AND SALT UPTAKE

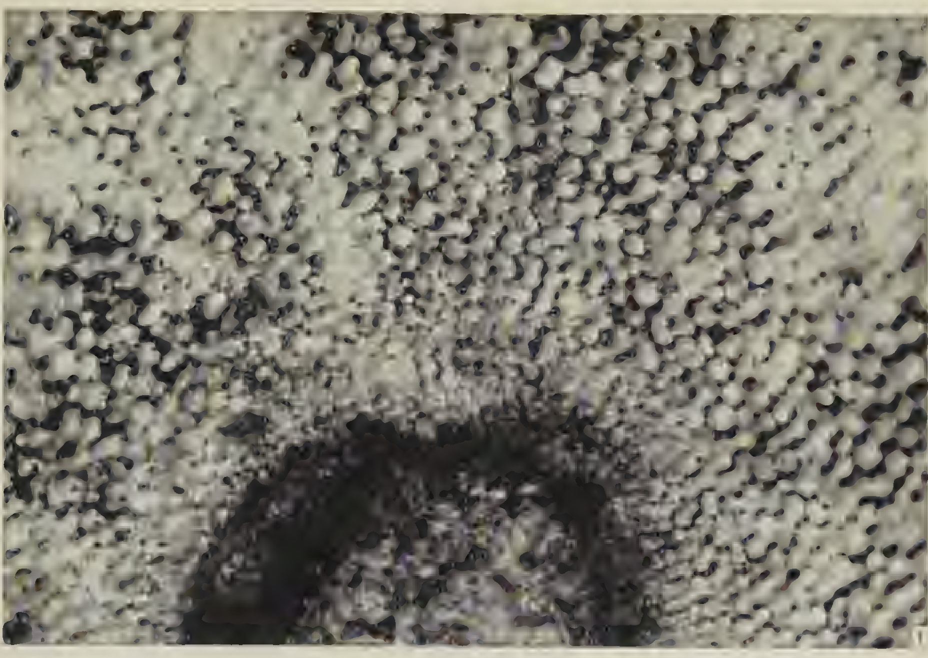

Fig. 1.—Photomicrograph of a fresh, hand-cut section of a bean root 3-5 mm. from the apex, under water. The dark spaces between the cells, particularly in the cortex, are air bubbles.

Fig. 2.—A second section, which was placed in a vacuum for 5 min., showing the absence of the dark spaces between the cells. Both approx. x109.

that the cation/anion mobility ratio for diffusion in protoplasm is increased compared with its value in water, and that the isopotential point of (at least the outside of) protoplasm occurs at a pH of 3-4 (Lundegardh 1940; Hope 1951) are also in accordance with this idea. Such a system would result in a Donnan distribution of ions between the protoplasmic and solution phases. It is proposed to test this by observing the effect on the size of the A.F.S. of varied salt concentration and varied pH. Robertson and Wilkins (unpublished data) have found that the initial uptake in carrot tissue is increased as a result of a decreased pH in the medium. This is consistent with a reduced concentration of immobile anions (due to de-ionization) allowing a larger concentration of mobile ions to be reached in the protoplasm.

Also, as suggested by Briggs and Robertson (1948), it should be possible to distinguish between diffusion in a phase containing immobile ions and that in one containing only mobile ones by modified experiments on the relation between p.d. and salt concentration. The p.d. *versus* salt concentration equation contains terms involving the concentration of immobile ions which reduce to the linear relation for simple diffusion p.d.'s when this concentration is zero. However, when the concentration of the immobile ion is not zero considerable deviations from equation (1) should occur.

V. Acknowledgments

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ON THE RATE OF COMPLETION OF DIAPAUSE DEVELOPMENT AT CONSTANT TEMPERATURES IN THE EGGS OF *GRYLLULUS COMMODUS* WALKER

By T. O. BROWNING*

[*Manuscript received December* 24, 1951]

Summary

The median duration of low-temperature treatment required by the eggs of *G. commodus* in order that they may develop and hatch without interruption due to diapause was least at 12.7°C., slightly longer or about the same at 15.9°C., and considerably longer at 10.3°C. At 8.5°C. it was not found possible to obtain 50 per cent. of the eggs free from diapause.

The temperature at which the eggs were incubated following exposure to low temperature greatly affected the influence of the low-temperature treatment. At 26.5°C. the median exposure to each low temperature necessary for the completion of diapause development was considerably shorter than that necessary when the eggs were incubated at 20.9°C., whilst when 29.9°C. was used as the incubation temperature, the median effective exposure to low temperature was still further reduced.

At 20.9°C. no eggs were found to complete morphogenesis and hatch without interruption when not first exposed to low temperature, whereas at 26.5°C. an average of 1 per cent. of the eggs did so and at 29.9°C. 26 per cent. of the eggs were found to hatch promptly.

The total numbers of eggs that hatched regardless of whether they experienced diapause or not were about the same at the two higher incubation temperatures but the totals hatching at 20.9°C. were significantly reduced.

Following low-temperature treatment the total numbers of eggs that hatched at all the incubation temperatures were greater the longer the exposure to low temperature they had received.

I. INTRODUCTION

The processes that go on during diapause resemble ordinary morphogenetic processes in that they exhibit a trend in the rate at which they are completed at different constant temperatures (Andrewartha 1944). For this reason they have been termed *diapause development* (Andrewartha 1952). The details of these processes are not known but in certain species it has been shown that they culminate in the elaboration of a hormone that evokes competence to develop (Williams 1948).

If eggs of *Gryllulus commodus* be incubated from the time they are laid at some temperature within their developmental range, most of them will develop for a few days and then enter diapause (Browning, unpublished data). But if newly laid eggs are first exposed to an adequate low temperature for an

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adequate period and then incubated, most of them will develop continuously and hatch promptly without the intervention of any diapause (Browning 1952*b*). In this *G. commodus* is unusual for with most species in which diapause occurs low-temperature treatment is most influential *after* the insect has entered diapause (Andrewartha 1943; Burdick 1937; Parker 1930). However, Salt (1952) indicates that in *Melanoplus bivittatus* eggs diapause development may be completed before the eggs have reached the morphogenetic stage at which diapause usually supervenes, so that development may continue through this stage without interruption. There is no reason to believe that the processes that occur in these two species during exposure to cold, resulting in their competence to continue their development without interruption, differ fundamentally from those occurring in other species when exposed to cold after diapause has become manifest. In what follows these processes will be referred to as "diapause development" even though the egg has not actually entered diapause at the time when they are going on.

Three other properties of *G. commodus* tend to complicate the investigation of diapause in this species:

(*a*) The proportion of eggs entering diapause depends upon the environment in which the female was living before she laid the eggs. For example, crickets collected from the same place in successive years laid eggs that differed in the proportion entering diapause when incubated at the same temperature.

(*b*) When batches of newly laid eggs were incubated at a series of temperatures within their developmental range, the proportion of the eggs that entered diapause varied with the temperature. A higher proportion of the eggs entered diapause when the temperature was low and relatively more developed without interruption at the higher temperatures. In this the eggs of *G. commodus* resemble the larvae of *Loxostege* (Steinberg and Kamensky 1936) or *Heliothis* (Ditman, Weiland, and Guill 1940).

(*c*) Because the proportion of eggs that exhibit diapause is dependent upon the temperature at which the eggs are incubated it is not feasible to specify the influence of low temperature on the completion of diapause independently of the temperature at which the eggs were subsequently incubated. This is perhaps most easily visualized if one considers that the egg incubated at a relatively low temperature has a powerful tendency to enter diapause and therefore may escape diapause only if it has previously been exposed for a long period to low temperature, during which time diapause development has been carried to an extreme. On the other hand, the egg incubated at a relatively high temperature has a weaker tendency towards diapause and may escape diapause even though it had experienced only a short exposure to low temperature, during which diapause development may not have been carried very far.

Thus a sample of eggs obtained in any one year will have a certain average propensity for diapause, depending upon the environment of the parents, and this may be modified by the temperature at which the eggs are incubated. However, if the eggs are stored at certain low temperatures the tendency

towards diapause may be eliminated from more or fewer of the eggs, depending on the duration of the low-temperature treatment and the temperature at which the eggs are incubated.

The aim of the experiment reported in this paper was to determine the range of temperatures over which diapause development could be completed successfully by the eggs of *G. commodus* and to determine which temperatures within this range were more favourable and which less favourable for its completion. At the same time the experiment aimed at determining whether the influence of low-temperature treatment was modified by the subsequent incubation temperature and at assessing the degree of interaction between the two influences.

II. Experimental Design and Methods

The experiment involves the estimation of the mean time required to be spent by the eggs of *G. commodus* at each of a series of low temperatures, in order that they may develop and hatch promptly when subsequently placed at some convenient temperature within their developmental range. This may conveniently and precisely be done by making use of the method of probit analysis (Finney 1947) to estimate the "median effective dose" (or E.D. 50), of low-temperature treatment, which in this case is the duration of exposure to a particular low temperature necessary to promote the completion of diapause in 50 per cent. of the individuals in a random sample of eggs.

The experiment was therefore designed to estimate the median effective exposure to low temperature in a sample of eggs and this was done at each of four different temperatures: 8.5°, 10.3°, 12.7°, 15.9°C. Five random samples of 150 newly laid eggs each were placed at each low temperature and removed to another thermostat for incubation after varying periods of exposure to cold. Four samples of 50 eggs were placed at the incubation temperature with no preliminary low-temperature treatment, to serve as controls.

At 8.5° and 15.9°C. the samples of 150 eggs were treated for either 10, 20, 25, 30, or 40 days; at 10.5°C. treatment was continued for 5, 10, 15, 20, and 30 days; whilst at 12.7°C. treatments of 5, 9, 13, 16, and 21 days were given. These particular durations were chosen because it was anticipated that they would result in a range of effectiveness on either side of the median, as measured by the percentage of the eggs that hatched promptly. It became evident that these were not the best durations to have chosen, largely because the lowest temperature was not so influential and the highest more influential than was anticipated.

When each lot of 150 eggs had been at low temperature for the appropriate time, it was subdivided into three random groups of 50 eggs each. One of these was incubated at 20.9°C., one at 26.5°C., and the third at 29.9°C. This was done because previous experience had shown that the temperature at which the eggs were incubated influenced the proportion that hatched with no appreciable interruption due to diapause.

When setting up the experiment 3600 eggs were obtained, within 24 hours of having been laid, from field-caught crickets by the method previously described (Browning 1952*b*). These were placed in random groups of 25 eggs each on damp plaster in small jars. Two jars were allotted to each treatment.

Following low-temperature treatment, the eggs remained at the incubation temperatures for varying periods. At 20.9°C. daily observations of hatchings were continued for 90 days, at 26.5°C. for 75 days, and at 29.9°C. for 60 days. This was done in an attempt to enable the same proportion, at each temperature, of all the eggs that were competent to hatch, to do so within the time allotted. When the period of incubation was complete the number of eggs in each jar that appeared alive and healthy was recorded.

Hatching in most treatments was spread unevenly over most of the period of incubation and in order to decide which eggs had developed with no appreciable delay due to diapause, reference was made to the distribution of hatching of diapause-free eggs from which the results given by Browning (1952*a*) were calculated. In this way it was decided that at 20.9°C. eggs that hatched between the 30th and 38th days of incubation inclusive were those in which diapause was complete or did not occur. Similarly, the limits at 26.5°C. were taken between 14 and 19 days inclusive, and at 29.9°C. between 10 and 14 days inclusive.

III. Results

The solid black rectangles in Figure 1 represent the numbers of eggs that hatched within the time required by diapause-free eggs at each of the incubation temperatures and it can be seen that an increased duration of exposure to low temperature in general resulted in an increase in the number of eggs that developed and hatched promptly. From these results the median effective duration of exposure to each low temperature was calculated for each incubation temperature, making allowance for the numbers of eggs that developed free from diapause among the controls. Where it was not possible to carry out a rigorous calculation, an estimate of the E.D. 50 was made by eye. This was necessary either because the duration of low-temperature treatment had not been long enough to promote diapause completion in at least 50 per cent. of one of the samples (e.g. 12.7°C. followed by 20.9°C.) or because the numbers of diapause-free eggs in the samples of an array were too erratic to enable a precise calculation to be made (e.g. 15.9°C. followed by 29.9°C.) or, in one case (15.9°C. followed by 26.5°C.) because following the shortest duration of low-temperature treatment more than 50 per cent. of the eggs hatched promptly. In some cases no estimate of the median effective duration of exposure to low temperature was possible because at the duration producing the greatest number of diapause-free eggs less than 50 per cent. hatched promptly, whilst further increase in the duration of low-temperature treatment resulted in a decrease in the numbers of diapause-free eggs. This occurred at all the incubation temperatures following exposure to 8.5°C. In these cases it seems that no duration of exposure to low temperature would result in 50 per cent. of

the eggs hatching promptly and so the median effective duration of low-temperature treatment becomes meaningless. Similarly there is no reason to suppose that an E.D. 50 existed for the eggs treated at 10.3°C. and incubated at 20.9°C. (Fig. 1).

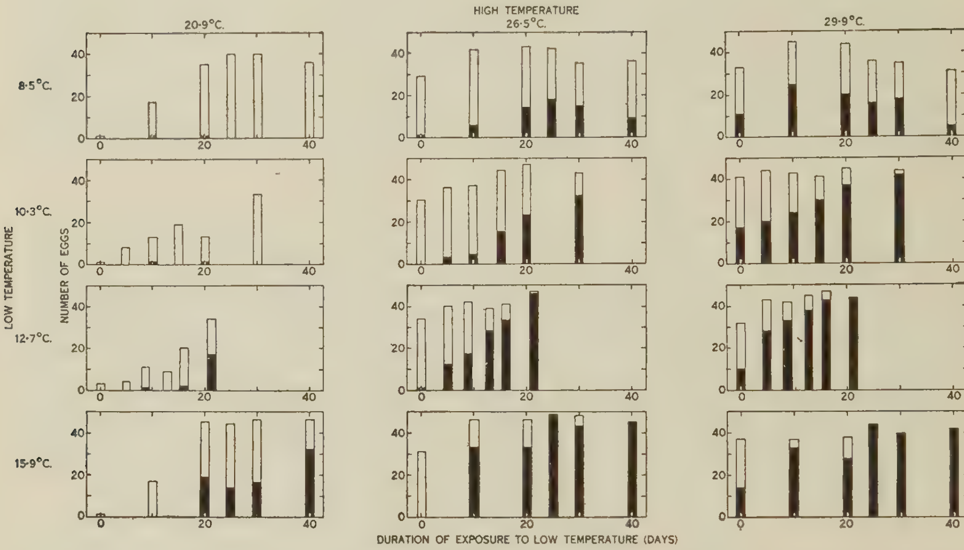

Fig. 1.—Histograms showing the total numbers of eggs that hatched in each treatment during the experiment (hollow rectangles) and the numbers of eggs in each treatment that hatched in the time required by diapause-free eggs at each particular incubation temperature (solid rectangles).

In Table 1 are set out the median durations of exposure to low temperature necessary to promote uninterrupted and prompt development of the eggs at each incubation temperature. These values may be converted to reciprocals and multiplied by 100 to give the percentage of diapause development completed per day, which is the most convenient form in which rates of morphogenetic development at constant temperatures may be represented (Davidson 1944). The results thus obtained are represented graphically in Figure 2. From the figure and Table 1 it can be seen that the rate of completion of diapause development at low temperature was dependent on the temperature used, being very slow at 8.5°C., increasing up to 12.7°C., and then becoming either less rapid or only slightly more rapid at 15.9°C. Such a trend in the rate of completion of diapause at low temperature has been demonstrated by Andrewartha (1943, 1944) and is implicit in a great deal of the work on the influence of temperature on the completion of diapause (e.g. Parker 1930).

From Table 1 and Figure 2 it can be seen that the influence exerted by any particular low temperature in inducing eggs to hatch without delay when subsequently incubated was dependent upon the temperature at which the eggs were incubated, being greatest at the highest of the incubation temperatures used. Indeed, under the conditions of the experiment, treatment at the

two lowest temperatures had no measurable influence on the completion of diapause when the eggs were incubated at 20.9°C. and at both 12.7° and 15.9°C. treatment had to be extended before any appreciable influence was discernible when the eggs were incubated at 20.9°C. When either of the other incubation temperatures was used all the low temperatures had a marked influence on reducing the proportion of eggs that entered diapause.

Temperature influenced the proportion of eggs among the controls (eggs that received no preliminary low-temperature treatment) that hatched without appreciable delay due to diapause (Fig. 1). Of the eggs placed immediately at 20.9°C., none was found to hatch without diapause; at 26.5°C. an average of 1 per cent. hatched promptly, whilst at 29.9°C. an average of 26 per cent. of the eggs hatched promptly. This response is similar to that of the larvae of *Loxostege* in which a greater proportion develop without diapause when reared at higher temperatures than at lower (Steinberg and Kamensky 1936).

TABLE 1

MEDIAN DURATION OF EXPOSURE TO EACH LOW TEMPERATURE (DAYS) NECESSARY TO PROMOTE PROMPT DEVELOPMENT AT EACH INCUBATION TEMPERATURE

Low Temperature (°C.)	Incubation Temperature 20.9°C.	26.5°C.	29.9°C.
8.5	—	—	—
10.3	—	22.9	14.5
12.7	23.4*	11.2	7.0
15.9	32.3*	10.5*	8.9*

A dash indicates that under these conditions an E.D. 50 is probably non-existent.

* E.D. 50 estimated by eye. Significant differences between E.D. 50's were estimated from the logarithms of the E.D. 50's and their standard deviations. Among the calculated E.D. 50's, 22.9 is significantly greater than 11.2 and 7.0 whilst both 11.2 and 14.5 are significantly greater than 7.0.

The incidence of eggs initially free from diapause seems to be influenced in some way by the environment of the parents during their active life, for in an experiment with the eggs of crickets caught in 1950 in the same area as those used in the present work (which were from the 1951 generation) and treated similarly in the laboratory, the following results were obtained: At 25.1°C., 5 per cent. of the eggs hatched with no appreciable delay due to diapause; at 27.0°C., 16 per cent. of the eggs hatched promptly; and at 29.0°C., 18 per cent., the difference between the first of these percentages and the other two being highly significant. Similarly in 1949, using crickets from the same area and similarly treated, an average of 23 per cent. of the eggs were found to be free from diapause when incubated at 26.8°C. (Browning 1952*b*).

In Figure 1 the total numbers of eggs that hatched during the period of observation are represented by the height of the hollow rectangles and it can be seen that, in general, increase in the duration of preliminary low-temperature treatment resulted in an increase in the numbers of eggs that hatched. At

8.5°C., however, prolonged low-temperature treatment resulted in a decrease in the number of eggs that hatched. However, when the numbers of apparently healthy eggs remaining in the jars at the end of the experiment were taken into account, there was no evidence of a significant increase in mortality among the eggs following the longer exposures to low temperature.

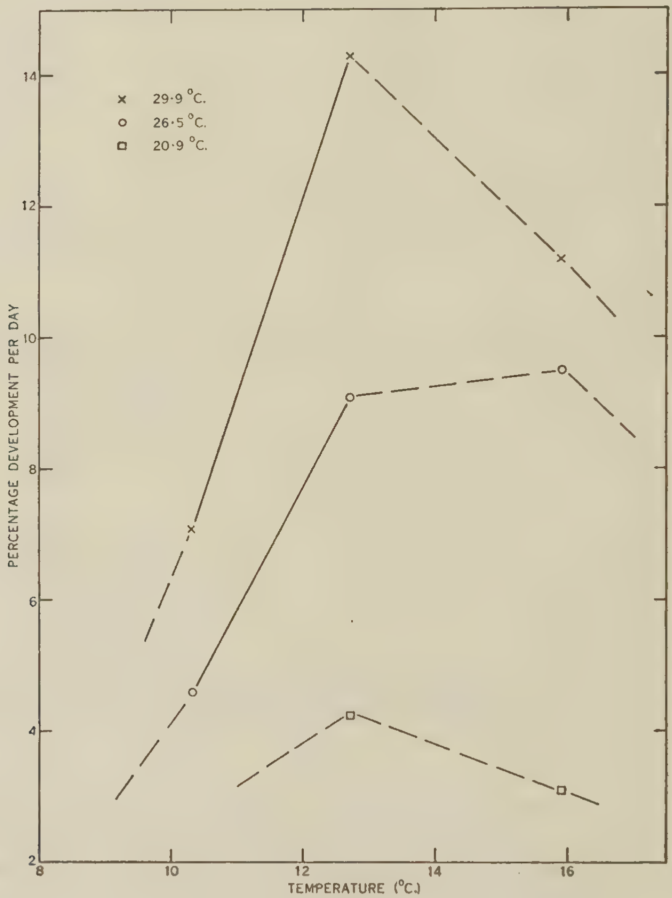

Fig. 2.—Freehand curves showing the trend in rate of completion of diapause development with change in temperature at each of the incubation temperatures. Continuous lines join points whose E.D. 50's were calculated and broken lines indicate points whose E.D. 50's were estimated by eye.

In Table 2 are shown the mean numbers of eggs that hatched during the whole period of observation following each temperature treatment, but without regard to the various durations of low-temperature treatment. The means were calculated from the totals in the two replicate sets of jars; since there were five durations at each low temperature, there were five jars in each set, giving a total of 125 eggs in each. In general, the numbers of eggs that

hatched at 26.5° and 29.9°C. were similar but these differed significantly from the numbers that hatched when the incubation temperature was 20.9°C. The interaction between the high-temperature and low-temperature treatments was highly significant as exemplified by the significant differences between the numbers that hatched at 20.9°C. following treatment at 8.5° and 15.9°C. on the one hand and 10.3° and 12.7°C. on the other.

TABLE 2

MEAN NUMBERS OF EGGS THAT HATCHED FROM 125 EGGS AT EACH INCUBATION TEMPERATURE FOLLOWING EXPOSURE TO EACH LOW TEMPERATURE

Incubation Temperature (°C.)	Low Temperature			
	8.5°C.	10.3°C.	12.7°C.	15.9°C.
20.9	84.5	43.5	40.5	99.5
26.5	113.5	118.5	121.5	132.5
29.9	112.0	129.0	126.5	119.0

A difference of 15.7 between any two means is significant, with a probability of 1 per cent.

IV. Discussion

The curves of Figure 2 show a progressive change with temperature in the rate at which diapause development was completed in the eggs of *G. commodus*, similar to that demonstrated in the eggs of *Austroicetes* (Andrewartha 1944). Such a trend is very similar to that demonstrable for most developmental processes, although the range of temperature is lower than that usual for other kinds of development. These results lend support to the concept that the completion of diapause is essentially a developmental process (Andrewartha 1952).

The inception of diapause in eggs that had experienced no preliminary low-temperature treatment was influenced by the temperature at which the eggs were incubated, more eggs hatching promptly at the highest than at the lower incubation temperatures. The same influence was probably exerted by the incubation temperature on eggs that had experienced low-temperature treatment, as evidenced by the influence of any particular duration of exposure to any low temperature on the subsequent development of the eggs being modified by the incubation temperature at which they were placed. For example, 30 days exposure to 10.3°C. resulted in 84 per cent. of the eggs hatching without interruption when incubated at 29.9°C., whereas following the same low-temperature treatment 64 per cent. hatched promptly at 26.5°C. and none at 20.9°C. In each case the average proportion of diapause development that had been completed by the eggs during treatment at low temperature would not differ significantly; yet more eggs hatched at the higher temperatures than at the lowest. In explanation of this, it would seem that an egg that is to be incubated at a particular temperature requires to have completed a particular amount of diapause development; this amount is greater if it is to be incubated at a lower temperature than that required if the incubation temperature is to be higher.

That the increased numbers of eggs that hatched during the whole period of observation following increased durations of low-temperature treatment (Fig. 1) was due to the influence of low temperature on the progress of diapause development in the eggs is exemplified by the low-temperature treatments that were unsuccessful in inducing eggs to hatch promptly at the incubation temperatures. Increase in the duration of exposure to 8.5°C. made little or no difference to the numbers of eggs that completed their diapause development to the point where they were competent to hatch without delay when incubated at 20.9°C. However, as the period of treatment at 8.5°C. was extended there was a progressive decrease in the mean time required for the completion of morphogenesis by eggs that did hatch at 20.9°C. (Table 3), and at the same time an increasing number of eggs hatched. This suggests that the influence of low-temperature treatment, although insufficient to enable diapause development to reach the point where morphogenesis could proceed uninterrupted at 20.9°C., was nevertheless sufficient to enable some eggs to complete the remainder of their diapause development concurrently with morphogenesis when placed at 20.9°C. and that the proportion of eggs competent to do this increased as the duration of treatment at low temperature increased.

TABLE 3

INCREASE IN PERCENTAGE HATCH AT 20.9°C. AND DECREASE IN MEAN TIME REQUIRED TO HATCH FOLLOWING INCREASED DURATION OF TREATMENT AT 8.5°C.

	Duration of Exposure to 8.5°C. (days)					
	0	10	20	25	30	40
Percentage hatching	2	34	70	80	80	72
Mean time required to develop and hatch (days)	84	74.5	64.8	65.0	60.4	54.4

V. Acknowledgments

The author is indebted to Dr. H. G. Andrewartha for his helpful advice and criticism and to Miss M. E. Morphett and Miss C. M. Hill for technical assistance.

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THE DEVELOPMENT OF PRONUCLEI IN THE RAT EGG, WITH PARTICULAR REFERENCE TO QUANTITATIVE RELATIONS

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Summary

The development of pronuclei has been studied by examining rat eggs freshly recovered at various times between ovulation and the first segmentation.

Pronuclear development is divided for convenience into six stages on the basis of the changes occurring in the male pronucleus. By noting the frequency with which the stages were observed the relative duration of each stage has been estimated.

The diameters of the nucleoli and pronuclei were measured at each stage of pronuclear development. The data include the numbers of nucleoli in each pronucleus, and calculations are made of the volumes of nucleoli and pronuclei, and the total surface areas of the nucleoli.

In the male pronucleus the mean number of nucleoli shows an early rapid fall from an initial figure of 7.5 to about one, and then a rise to a level of about 17. On the other hand the mean number of nucleoli in the female pronucleus increases steadily from about three in the first stage to nine in the final stage.

The volumes of the pronuclei increase to reach a maximum in the second half of pronuclear development. The final volume of the male pronucleus lies between 5000 and 6000 cu. μ and that of the female between 2000 and 2500 cu. μ.

The total volume of the nucleoli in each pronucleus increases rapidly to achieve a level in the first third of pronuclear development which is maintained for the remaining two-thirds. In the male pronucleus the maximum total nucleolar volume was about 550 cu. μ, and in the female about 220 cu. μ.

The total surface area of the nucleoli reaches a maximum more rapidly in the female pronucleus than in the male, the difference being referable to the formation of a single nucleolus at an early stage in the male. Maximum values for the total surface area of nucleoli were about 730 sq. μ in the male pronucleus, and about 345 sq. μ in the female.

I. Introduction

The earliest detailed description of the changes shown by the pronuclei of the mammalian egg is probably to be found in the classical paper by Sobotta (1895) on the mouse egg. According to Sobotta, the early pronuclei contain chromatin in dense strands with irregular nodal thickenings; later the chromatin is formed into nucleoli. He considered that the male pronucleus always has only one large nucleolus, the female either one or, more commonly, several.

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Sobotta noted that, with further development, the nucleoli begin to stain lighter and the chromatin breaks up and becomes irregularly scattered on the achromatic network. Finally the nucleoli become clear sacs, only their outlines remaining.

Lams and Doorme (1908), who also investigated the mouse egg, noted that the early nucleoli have a transitory existence; at a certain stage the chromatin contained in the nucleoli undergoes fragmentation into little masses situated closely in contact with the nuclear membrane and along fine filaments. They observed that these filaments seem to be disposed more or less radially about a large "plasmatic nucleolus" which is always excentric in position. At a later stage the chromatic granulations were found to exist in larger numbers, but the nucleolus was always non-staining and homogeneous.

In the rat egg, Sobotta and Burckhard (1910) observed changes broadly similar to those Sobotta had described for the mouse egg: the pronuclei in the early stages contain chromatin masses and a linin network; later the nucleoli disappear and a very distinct network is present instead.

The pronuclei of the guinea pig egg were found by Lams (1913) to contain a fine reticulum, between the threads of which were granules and chromatic globules, rodlets, and "nucleinic" nucleoli. According to this author the nucleoli initially are clearly "nucleinic"; towards the time when the chromosomes of the first segmentation spindle become visible the nucleoli are "plasmatic."

Kremer (1924) re-examined Sobotta's preparations of mouse and rat eggs and attempted a logical account of the growth and development of pronuclei. He maintained that pronuclear growth could be divided into two stages: a stage of chromatin increase, and a stage of chromatin reduction. In the first stage the early pronuclei contain small chromatic masses or globules of varying size, some of which take the iron haematoxylin stain more weakly than others and some show outer dark and inner clear zones. Kremer believed that the globules grow and coalesce, drawing material from the cytoplasm, until a single large nucleolus, the "principal nucleolus," is formed. The second stage, which he described, is characterized by a reduction in the accumulated chromatic substance: the "principal nucleolus" loses its staining affinity, the chromatin is split up and, in the form of fragments, is passed back into the cytoplasm. Kremer observed chromatic bodies, resembling small nucleoli, lying scattered through the cytoplasm and considered these to be the nucleolar fragments that had migrated from the pronucleus.

Kremer's theory was that the pronuclear bodies are synthesized from ooplasmic material and, in the pronuclei, are brought into close chemical relations with the specific maternal and paternal nuclear constituents. After the nuclear chromatin has reached its greatest accumulation the now adequately treated substances leave the pronuclei in the form of chromatic fragments. Kremer suggested that each of these may be regarded as a carrier of maternal or paternal hereditary qualities, which are thus distributed over the whole egg and pass equally into all descendant cells.

Mainland (1930) considered that the pronuclei of the ferret egg pass from a condition of uniformly stained chromatic material to one in which this

material is in the form of globules and granules of various sizes, the larger and medium-sized particles occurring at later periods. Occasionally he saw, even in an early pronucleus, a more or less spherical body with a darkly stained periphery and a pale centre. In general, eosinophilic, pale, or colourless particles were much more commonly found in the later central than in the earlier peripheral pronuclei. Mainland could find no evidence of a removal of chromatin from the pronuclei such as Kremer claimed.

In the mouse egg, Gresson (1942) noted that the male pronucleus early contains a single nucleolus but with the rapid increase in size of the pronucleus several nucleoli appear. The female pronucleus, on the other hand, had several nucleoli, sometimes as many as 10 or 11. In later states, according to Gresson, the pronuclei are about the same size and have the same number of nucleoli.

All these investigators made their observations on histological material and it is apparent that a concept of pronuclear development based upon their descriptions is both incomplete and confusing. A proper understanding of the nature and significance of the fertilization process requires an accurate knowledge of pronuclear development, but it seems unlikely that this can be obtained from material prepared by the older histological methods. A new approach to the problem has recently been attempted by examining living eggs with the phase-contrast microscope (Austin 1951). Observations described in that paper were made chiefly on the process of fertilization as it took place *in vitro*. The present paper concerns the study of freshly recovered eggs, which were examined with the object of determining the form of the changes occurring *in vivo* and of obtaining quantitative data on the development of the pronuclei.

II. Methods and Materials

The materials and most of the procedures employed were similar to those described in a previous communication (Austin 1951). In the present investigations, however, eggs were recovered from the fallopian tubes in saline and not under paraffin as they were not required for protracted observation.

III. Observations

(*a*) *General Course of Pronuclear Development*

On the basis of experience previously described (Austin 1951), and that obtained subsequently, it has been found convenient to consider the process of pronuclear development as passing through six consecutive stages. These have been named, respectively, early and late primary growth, single nucleolus stage, early and late secondary growth, and, finally, pronucleus at full development. These stages are based upon the changes shown by the male pronucleus, which are much more distinct than those occurring in the female pronucleus.

Early primary growth begins with the first appearance of "primary" nucleoli in the structure representing the metamorphosed sperm head, and includes the initial enlargement of these nucleoli (Plate 1, Fig. 1). The nucleoli

show further growth during the late primary growth stage (Plate 1, Fig. 2) and also undergo coalescence so that generally a single large primary nucleolus is formed.

For a period the large primary nucleolus continues to enlarge at a rate seemingly parallel to that at which the pronucleus enlarges, but later the pronucleus begins to grow more quickly. This is referred to as the single nucleolus stage (Plate 1, Fig. 3).

In the early secondary growth stage (Plate 1, Fig. 4) the rate of growth of the pronucleus clearly exceeds that of the primary nucleolus and more free space becomes apparent within the pronucleus. Soon new nucleoli make their appearance at the periphery of the pronucleus; these are called the "secondary" nucleoli. As they grow the secondary nucleoli begin to move away from the periphery.

The stage of late secondary growth is characterized by the still larger size of the pronucleus and by the presence of a number of secondary nucleoli distributed both on the nuclear membrane and nearer the centre of the pronucleus (Plate 1, Fig. 5). The large primary nucleolus undergoes a distinct reduction in size during this stage.

In the pronuclei that have reached the stage of full development there is a larger number of secondary nucleoli and many of these have acquired a moderate size (Plate 1, Fig. 6). The single large primary nucleolus has become still smaller so that it is now not much larger than the secondary nucleoli. At this stage the nucleoli are grouped in the central region of the pronucleus, away from the nuclear membrane.

The development of the female pronucleus differs from that of the male. The nucleoli make their appearance from the chromosome group at the vitelline end of the second maturation spindle. As they grow the nucleoli undergo little coalescence so that rarely is a single nucleolus formed. Secondary nucleoli begin to appear earlier than in the male pronucleus but the total number of nucleoli ultimately found is only about half that of the male pronucleus.

In the male pronucleus the nucleoli lie, in the main, away from the nuclear membrane, except during early and late secondary growth, particularly the former, when secondary nucleoli are found on the nuclear membrane and may even be slightly flattened against it. The nucleoli of the female pronucleus, on the other hand, are mostly to be seen near the nuclear membrane throughout development. In the stages of growth that correspond to late primary growth, single nucleolus, and early secondary growth of the male pronucleus the nucleoli in the female pronucleus are often found deeply embedded in the nuclear membrane (Plate 1, Fig. 7). For the whole period of development the male pronucleus is larger than the female.

The stage of full development is terminated by the rapid reduction of the pronuclei and nucleoli, the disappearance of the nuclear membrane, and finally the dissolution of the nucleoli to give place to the prophase chromosomes of the first segmentation mitosis. The details of these changes as they were

seen to occur *in vitro* have been described (Austin 1951). Examination of freshly recovered eggs at the appropriate stages has indicated that the process is essentially the same *in vivo*.

(*b*) *Relative Duration of Stages in Pronuclear Development*

To obtain a measure of the relative duration of the developmental stages described in the last section, mature female rats were placed with males in the evening and examined the following morning for evidence of mating. Rats with copulation plugs were killed at 6-hourly intervals from 8.0 a.m. on the first day to 8.0 a.m. on the following day, and the eggs were examined to determine the stage of fertilization. Data were obtained from five rats at each of the times indicated (Table 1).

TABLE 1

NUMBER OF EGGS IN DIFFERENT STAGES OF FERTILIZATION WHEN FRESHLY RECOVERED FROM RATS KILLED AT VARIOUS TIMES DURING THE DAY AND NIGHT FOLLOWING MATING

Hour	Number of Rats Used	Number of Unpenetrated Eggs	Number of Eggs in Pre-Pronuclear Stages	Number of Eggs in Pronuclear Stages: Primary Growth	Single Nucleolus	Early Secondary Growth	Late Secondary Growth	Full Development	Number of Eggs in Post-Pronuclear Stages	Total Eggs
8.0 a.m.	5	3	42	6	1	0	0	0	0	52
2.0 p.m.	5	0	0	12	10	17	11	0	0	50
8.0 p.m.	5	3	1	0	0	11	12	19	0	46
2.0 a.m.	5	1	0	0	0	0	2	30	12	45
8.0 a.m.	5	1	0	0	0	0	0	7	41	49
Totals	25	8	43	18	11	28	25	56	53	242

Most (45 out of 52) of the eggs obtained from the rats killed at 8.0 a.m. on the first day either had no sperm within (three eggs) or were in the stages of fertilization preceding pronucleus formation (42 eggs). There were, however, six eggs with pronuclei in primary growth, and one in the single nucleolus stage. Rats killed at 2.0 p.m. yielded 50 eggs, all with pronuclei; in 11 of these the pronuclei had reached the stage of late secondary growth. At 8.0 p.m., three eggs were unpenetrated and one had a sperm in the perivitelline space, the remaining 42 eggs having pronuclei in the stages of early and late secondary growth, and full development, principally the last-named. At 2.0 a.m. the following morning most of the eggs (30 out of 45) were in the stage of full development and 12 were either in the segmentation stages or had already divided; one egg was unpenetrated.

In order to obtain from these data the approximate duration of each of the pronuclear stages, two methods may be used. By the first method the relative duration is assumed to be proportional to the frequency with which each stage is observed. This is the more direct method but is open to the objection that the rats were killed at set intervals and not at random times,

thus possibly favouring certain pronuclear stages. However, the results so obtained are essentially the same as those that can be derived by the use of the second method, the calculation of the cumulative percentages of eggs passing through each stage. Because of its simplicity the former method is followed here.

The total number of eggs having pronuclei in each of the stages of primary growth, single nucleolus, early and late secondary growth, and full development were 18, 11, 28, 25, and 56 respectively. It can be seen that the single nucleolus stage is the shortest, that early and late secondary growth are each about two and a half times as long and full development about five times as long. Primary growth is subdivided into early and late phases of approximately equal duration, each of which would be a little shorter than the single nucleolus stage. This information is used below for the expression of the approximate rates of dimensional change in pronuclei and nucleoli during the course of pronuclear development.

(*c*) *Quantitative Changes in Pronuclear Development*

Adult female rats were placed with males in the evening and examined the following morning. Those with copulation plugs were killed at times varying from 8.0 a.m. the same day to 8.0 a.m. the following day. In this way eggs were obtained with pronuclei in all the stages described in Section (*a*) above.

The eggs recovered were classified according to the stage of pronuclear development. The diameter of each nucleolus, and the longest and shortest horizontal diameters of the pronuclei, were measured with an eyepiece micrometer. The vertical depth of each pronucleus was estimated with the aid of the calibrated fine adjustment on the microscope.

Altogether 96 eggs were examined in this way and the following information was obtained:

(i) *Number of Nucleoli* (Table 3).—The number of nucleoli in the female pronucleus showed a steady increase from a mean of three in the early primary growth stage to a mean of about nine at full development (Fig. 1*A*). In this series a single nucleolus was not seen in the female pronucleus, but in two eggs there were only two nucleoli in each of the female pronuclei. The male pronuclei of these eggs were at the single nucleolus stage.

The mean number of nucleoli in the male pronuclei, initially between seven and eight, showed a steep fall to the single nucleolus stage. In only one egg out of 14 at this stage was there more than one primary nucleolus seen and this egg had two. During the secondary growth phase the mean number of nucleoli rapidly increased to reach a maximum of about 17 (Fig. 1*A*).

(ii) *Volumes of Pronuclei and Nucleoli* (Table 2).—The volumes of both male and female pronuclei increased slowly in the primary growth phase, more rapidly during early secondary growth, and then more slowly to form a plateau at the stage of full development (Fig. 1*B*). At maximum size the mean volume

TABLE 3

NUMBERS AND TOTAL SURFACE AREAS OF NUCLEOLI AND VOLUMES OF LARGEST NUCLEOLI AT VARIOUS STAGES OF PRONUCLEAR DEVELOPMENT

Stage of Pronuclear Development	No. of Eggs Examined	Nucleoli of Male Pronucleus: Number of Nucleoli		Total Surface Area of Nucleoli (sq. μ)		Volume of Largest Nucleolus (cu. μ)		Nucleoli of Female Pronucleus: Number of Nucleoli		Total Surface Area of Nucleoli (sq. μ)		Volume of Largest Nucleolus (cu. μ)	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Early primary	4	4-9	7.5 ± 1.19*	77-163	107.8 ± 26.80*	6-131	39.3 ± 30.60*	0-6	3.0 ± 1.29*	0-87	45.0 ± 20.01*	0-48	16.25 ± 11.20*
Late primary	9	2-12	5.9 ± 1.03	220-353	280.0 ± 15.77	35-326	136.8 ± 36.52	2-7	4.6 ± 0.64	91-201	138.6 ± 13.32	16-104	47.4 ± 9.43
Single nucleolus	14	1-2	1.07 ± 0.07	206-337	273.4 ± 10.04	253-579	423.1 ± 23.91	2-8	5.3 ± 0.55	54-291	203.4 ± 16.14	35-161	83.5 ± 10.62
Early secondary	26	1-11	4.7 ± 0.59	254-646	427.3 ± 19.09	234-839	456.1 ± 32.42	3-9	6.9 ± 0.33	201-381	287.8 ± 9.61	48-326	112.1 ± 11.60
Late secondary	26	4-30	17.5 ± 1.37	485-910	660.9 ± 21.26	48-579	280.5 ± 31.84	3-14	7.5 ± 0.47	227-433	321.8 ± 9.98	35-195	98.8 ± 8.46
Full development	17	5-36	17.1 ± 2.23	481-909	730.1 ± 30.79	82-442	203.8 ± 20.84	5-12	9.0 ± 0.49	234-390	345.2 ± 9.57	35-104	65.4 ± 6.02

* Standard error.

The volumes then remained constant throughout the stages of late secondary growth and full development. The final volume achieved by the nucleoli of the male pronuclei, about 550 cu. μ, was twice that of the nucleoli of the female pronuclei, about 225 cu. μ. During the initial phase of growth the volume of the male nucleoli increased at a greater rate than that of the female nucleoli.

The mean volume of the largest nucleolus in the male and female pronuclei was also calculated (Table 3, Fig. 1*D*). In the female pronuclei the volume of the largest nucleolus increased fairly rapidly to a maximum of about 100 cu. μ at the early secondary stage and then declined slowly to about 60 cu. μ at the stage of full development. The volume of the largest nucleolus in the male pronuclei showed a much more rapid increase during primary growth and reached a maximum of about 450 cu. μ in the early secondary growth stage. Later, particularly during late secondary growth, the volume decreased to a terminal value of about 200 cu. μ.

(iii) *Surface Area of Nucleoli* (Table 3).—The total surface areas of the nucleoli increased throughout pronuclear development and in much the same general manner in both male and female pronuclei (Fig. 1*E*). The increase was most rapid in the early stages. The final surface area achieved by the nucleoli in the male pronucleus, about 730 sq. μ, was twice as large as in the female pronucleus, about 350 sq. μ.

The curve for the nucleolar surface area in the male pronucleus showed a distinct discursion with the formation of the single nucleolus, but the increase in surface area was resumed during the stage of early secondary growth.

IV. Discussion

The observations on freshly recovered eggs described in this paper indicate that the steps involved in the early phase of pronuclear development *in vivo* are essentially the same as those observed *in vitro* and described in a previous communication (Austin 1951). The only notable difference lies in the evidence that a single nucleolus is not usually formed in the female pronucleus *in vivo,* whereas it has been observed to form in several eggs studied *in vitro*.

It seems likely that the regular formation of a single nucleolus in the male pronucleus can be ascribed to limitation of space. Contact between nucleoli generally results in coalescence, and nucleolar volume increases so rapidly during the phase of late primary growth that the pronucleus becomes incapable of accommodating two or more nucleoli without their coming in contact with one another. This applies rather more to the male pronucleus for, although the male and female pronuclei grow at about the same rate, nucleolar volume increases more rapidly in the male.

The general pattern of pronuclear development, involving the formation of a single large nucleolus from a number of small nucleoli, and its later replacement by a number of medium-sized nucleoli, is in agreement with the observations of previous workers. However, by examining living eggs it has been possible to obtain further information, including quantitative data, on the changes that take place during pronuclear development.

The total volume of the nucleoli in both pronuclei is found to increase rapidly from their first appearance until a maximum is reached in the early secondary growth stage. This level is maintained until the terminal sudden decrease in volume which occurs when the nucleoli give place to the prophase chromosomes of the first segmentation mitosis.

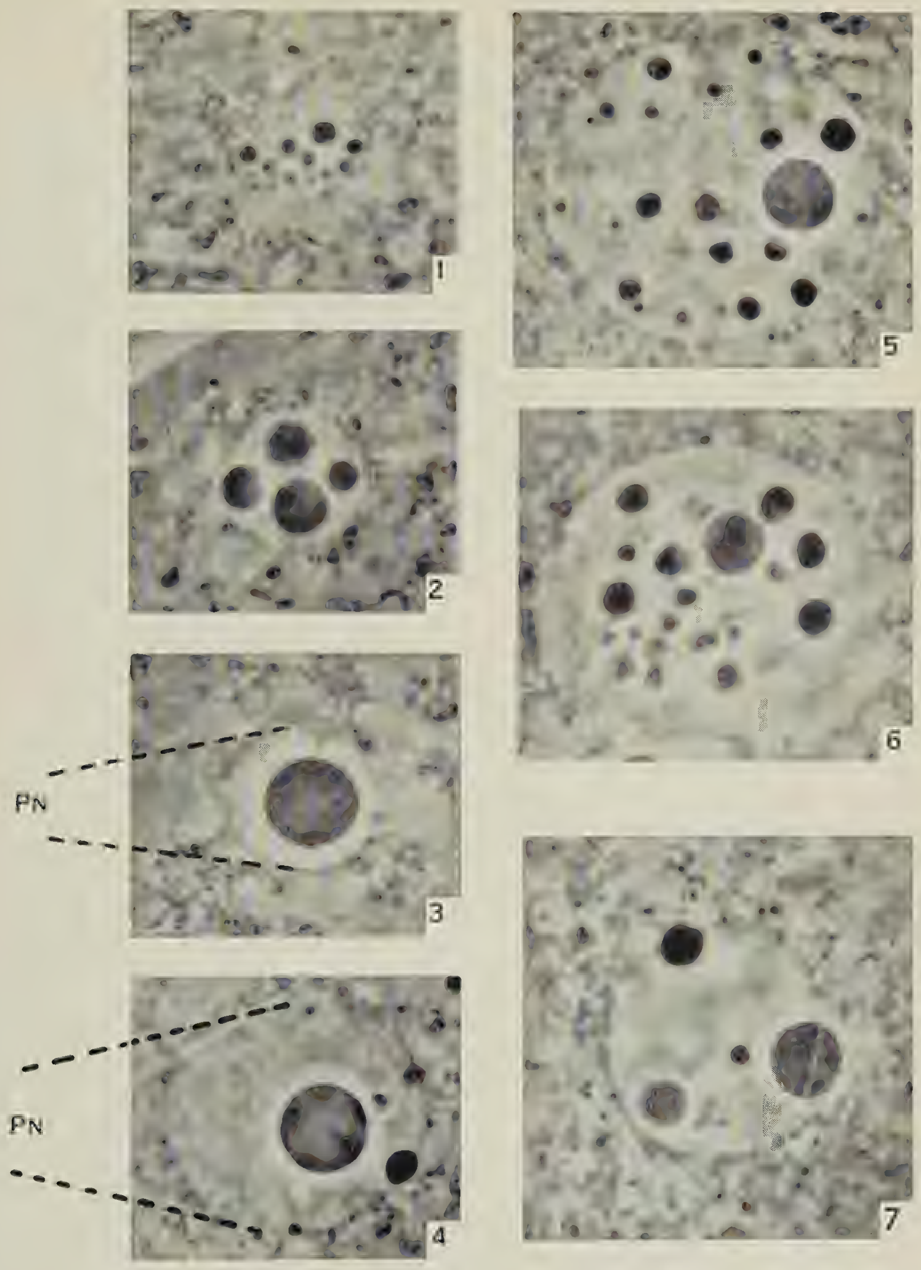
Although the volume remains constant the number of nucleoli continues to increase after the early secondary growth stage. This is accompanied in both pronuclei by a reduction in size of the largest nucleoli and the growth of the newly formed secondary nucleoli; the change is apparent in the male pronucleus much more than in the female. There is thus a redistribution of nucleolar material during the secondary growth stages. This does not appear to take place by a process of fragmentation as Kremer (1924) suggests, for if fragmentation were involved the secondary nucleoli would surely be found first in the near vicinity of the large primary nucleolus; on the contrary they regularly occur near the periphery of the pronucleus. The indications are, in fact, that the secondary nucleoli originate on the nuclear membrane. There is no evidence for the migration of nucleoli out of the nucleus and into the cytoplasm; rather the movement is towards the centre of the nucleus.

As expected from the other observations, the total surface area of the nucleoli was found to increase throughout pronuclear development even though total nucleolar volume remained constant for most of this period.

Seen through the phase-contrast microscope, the nucleoli appear as smooth, uniformly black spheres that differ from each other only in size. On the other hand, most of the previous investigators have described differences in the staining properties of the nucleoli, which suggests that there exist corresponding differences in the chemical nature of the nucleoli. It seems more likely, however, that the effects observed are referable to weakly or negatively basophilic nucleoli surrounded by a layer of strongly basophilic material which is present in limited total quantity. In these circumstances the appearance of the nucleoli in histological preparations would vary with the plane of section and with the effects of fixatives. Nucleoli with pale centre and darkly stained periphery have been described by both Kremer (1924) and Mainland (1930). In the later stages of pronuclear development a large proportion of lightly stained or unstained nucleoli would be expected, because of the increase in surface area, and such a distribution has been recorded by Mainland.

These ideas are consistent with the concept of the nucleolus and its surrounding layer of associated chromatin that has been developed by Caspersson and his associates (Caspersson 1950). It is not possible, however, to determine whether the same system exists in rat pronuclei by examining living eggs with the phase-contrast microscope. Information from more specific methods is required and it is proposed to further these investigations with the aid of the Feulgen reaction and ultraviolet microscopy.

DEVELOPMENT OF PRONUCLEI IN THE RAT EGG



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Explanation of Plate 1

The photographs were taken with the aid of a phase-contrast microscope. Magnification is x1400 for Figures 1-6, and x1800 for Figure 7.

Figures 1-6 illustrate the male pronucleus in the stages of pronuclear development referred to in the text.

Fig. 1.—Early primary growth.

Fig. 2.—Late primary growth.

Fig. 3.—Single nucleolus stage. Limits of pronucleus indicated (Pn).

Fig. 4.—Early secondary growth. Limits of pronucleus indicated (Pn).

Fig. 5.—Late secondary growth.

Fig. 6.—Full development.

Fig. 7.—Female pronucleus showing nucleoli embedded in the nuclear membrane.

THE FINE STRUCTURE AND BIOSYNTHESIS OF SILK FIBROIN

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Summary

Electron microscopic examination of fibrillar fragments produced by the enzymic disintegration of silk fibroin suggests the existence of fine microfibrils about 100 Å in diameter extended parallel to the length of the fibre axis. The microfibrils are similar in width to the crystalline micelles deduced from X-ray diffraction.

Fibroinogen, the soluble precursor of fibrous fibroin extracted from the mature silk glands of the silk-worm, spontaneously separates from dilute solutions in the form of fine microfibrils. This phenomenon appears to be similar to the formation of F-actin from G-actin and fibrin from fibrinogen, and is probably due to an unsymmetrical aggregation of the molecules of the soluble form.

The "soluble silk" of Coleman and Howitt (1947) does not possess the same property of spontaneously fibrillating as does natural fibroinogen. Assuming that a similar phenomenon occurs during the spinning of silk by the silk-worm, a possible course of events during spinning is outlined.

I. Introduction

Many natural fibres are characterized by an ordered structure extending from the molecular level to the macroscopic and the concept of levels of organization is a valuable aid to their description. A particular interest is the description of the fibre at the level of fine structure since structure at this level may be related to the orderly processes of biosynthesis which brought the fibre into being, and thus provides necessary information for an understanding of these processes. The term "fine structure" is used here in the sense advocated by Picken (1940) to describe structure with a dimension in the range *c.* 50-100 Å and at present most conveniently studied by electron microscopy. A distinction has been made between "spun fibres" and "grown fibres" largely on the basis of their fine structure (Frey-Wyssling 1948). Grown fibres, such as cellulose or keratin, possess a fine structure of microfibrils; whereas this feature is thought to be lacking in a spun fibre such as rayon.

Since there are obvious analogies between the extrusion and drawing of an artificial rayon fibre and the spinning of silk by the silkworm, it is natural to assume that silk is a "spun fibre" in the sense that it possesses little fine structure beyond the micelles indicated by X-ray diffraction. However, this question can only be answered by an actual search for fine structure by electron microscopy or other means. Previous work appeared to show the absence of definite microfibrils (Hegetschweiler 1950; Zahn 1949).

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In this paper two pieces of evidence will be advanced to show that silk fibroin may actually possess a fine structure of microfibrils similar to that possessed by other grown fibres. The first evidence results from an electron microscopic examination of disintegrated silk fibre; the second, less direct, comes from examination of the spontaneous fibrillation of the precursor protein extracted from the mature silk glands of the larvae of the silk-worm, *Bombyx mori* L. The biosynthesis of the silk fibre will be discussed in terms of these findings. Regenerated fibroin made by the method of Coleman and Howitt (1947) has also been examined for fine structure.

II. Experimental

(*a*) *The Existence of Microfibrils in Natural Silk Fibroin*

Silk fibres are too thick to be examined directly by the electron microscope and, as with other insoluble natural fibres such as hair, it is difficult to reduce them to fragments small enough for examination without destroying the evidence of fine structure. It is usually found with hair and the cellulosic fibres that mechanical disintegration produces fibrillar fragments of a great range of widths related rather to the severity of treatment than to fundamental structure; but on the other hand appropriate enzymic disintegration of hair reveals definite macro- and microfibrils and the study of plant cell walls and slimes demonstrates the reality of cellulosic microfibrils (Frey-Wyssling 1950; Preston *et al.* 1948; Wardrop 1949). The first experiments with silk fibres, made with fragments of acid-swollen silk disintegrated in a Waring Blendor, led to a similar mixture of fibrillar fragments of many sizes in the electron microscope. These findings seem to be essentially the same as those reported by Frey-Wyssling (1950), Hegetschweiler (1950), and Zahn (1949).

However, such a result was not unexpected when regard was taken of the consequence of desiccation and the possibility of strong lateral bonding between fibrillar elements, assuming these exist. It was therefore decided, following the experience with wool, to partially digest fibres by means of an enzyme and to examine the residue after mechanical breakdown.

Cocoon silk was reeled off immediately after spinning ceased, chopped into lengths of about 2-3 mm., and washed in several changes of 0.05 per cent. sodium oleate solution at 70°C. to remove the sericin. The de-gummed silk was rinsed in distilled water and then extracted for one day in a Soxhlet extractor using light petroleum b.p. 60-70°C. to remove fatty substances. Purified fibre (2 g.) was then added to 500 ml. of a trypsin solution (a Difco preparation 0.05 per cent., pH 8-8.6) and maintained at 40°C. for 6 months. The trypsin solution was changed once a week over this period. On several occasions the solution became infected with bacteria, which may have assisted the digestion.

At the end of this period the fibre was much damaged, and vigorous treatment in the Waring Blendor produced a suspension of fine fibrillar fragments. By fractional centrifugation the finer material was separated and re-

suspended in distilled water. Drops of this suspension were dried directly on collodion films and lightly shadowed with uranium in the conventional way.

Electron microscopic examination revealed fibrillar material of great diversity of size, much as has been already described. Attention was concentrated on the thinner ribbon-like fragments, in which fine structural details were most likely to be visible. The existence of what seem to be long, thin, parallel microfibrils was evident in micrographs such as Plate 1. It is difficult to measure the widths of fibrils laterally shadowed, but assuming those in Plate 1 are close-packed laterally, a width of not greater than 100 Å can be deduced.

Zahn (1949) has reported fibrils of 90 Å diameter without attributing special significance to them. To judge from his electron micrographs his preparation bears little resemblance to that described here. Hegetschweiler's (1950) preparation is more similar but this author also concluded that no definite microfibrils existed. As mentioned above these observations on silk are similar to those made on cellulose. The earlier electron microscopy of cellulose was carried out on mechanically disintegrated material and fine fibrils of a considerable range of diameter were found. When appropriate material, such as plant cell walls or slimes, was examined, discrete fine fibrils were discovered. It now seems probable that in both materials definite microfibrils are the primary structure and that these may be split longitudinally to yield finer fibrils.

The visual evidence of fine fibrils in silk fibroin provided by electron micrographs, such as that of Plate 1, can be criticized on the grounds that prolonged enzymic digestion may have been responsible for the result. The same criticism could be brought against similar findings concerning hair. Since it is impossible to effect disintegration of these resistant fibres in any other way without obviously damaging the structure, it is possible to meet the criticism only by stressing the apparent individuality of the microfibrils or by finding independent evidence of their existence. Such evidence may be found by new techniques in electron microscopy or by low-angle scattering of X-rays, but is not available at present. Low-angle discrete X-ray reflections indicating lateral spacings of the order of 100 Å have not been observed, but the existence of long, thin, crystalline micelles has been postulated to explain the observed size of X-ray reflections in the wide-angle pattern. The micelle predicted from X-ray work and the microfibril found by electron microscopy are of a similar order of width and may well be the same structure.

Actually the diameter of the microfibrils seems to be rather larger than the breadth of micelles computed from X-ray measurements and they are a great deal longer. However, the apparent differences in size may be merely a result of experimental difficulties. For instance, since not much reliance can be placed on measurements made on micrographs of metal-shadowed detail *c.* 100 Å in width, the diameter of the microfibrils may be somewhat less than 100 Å; on the other hand, X-ray estimates of micellar width are likely to be underestimates, since factors other than micellar size contribute to a broadening of X-ray reflections. Should the diameter of microfibrils prove beyond question to be greater than the micellar breadth, as may be the case if some estimates (Preston *et al.* 1948) of fibrillar width are correct, it will be necessary

to assume that within the microfibrils, which are assumed to be the original structures related directly to synthesis, a secondary crystallization of chains occurs leading to the "fringed micelles" of earlier writers (Meyer 1942).

From the occurrence of flattish sheets of microfibrils one might infer a preferred tendency towards unilateral association, but there was no evidence that these fine fibrils were grouped into definite macrofibrils as in the keratins.

(*b*) *The Spontaneous Formation of Fine Fibrils in Extracts of the Silk Gland of* B. mori

Certain aspects of the formation of silk fibre by the silk-worm are familiar and the analogy to the spinning of artificial fibres is often referred to. The proteins, which are the precursors of the insoluble fibroin and sericin, accumulate in solution in the silk gland as a result of the activity of the sericinogenic cells lining the gland (Bergmann 1939). In this article we are not concerned with the primary synthesis of proteins by these cells although they, with their remarkable nuclei, would repay further study by modern methods. The present problem concerns solely the mechanism whereby the soluble proteins, secreted by the cells, are converted into the insoluble fibre during the spinning of the thread of the cocoon.

The protein concentration in the gland is very high (about 30 per cent. of the fresh weight) and the solution is in a state akin to supersaturation. Mechanical disturbances such as stretching or flattening readily provoke the transformation into the insoluble form. The stretched gland contents yield a good fibre-type X-ray pattern identical with that of silk fibre (Meyer 1942). The untreated dried gland gives a pattern equivalent to that of disoriented fibre (Trogus and Hess 1933). Experiments have been in progress in this laboratory concerning changes in the X-ray pattern occurring during transformation. However, these need not be reported, since similar experiments have been published (Shimizu 1941; Kratky, Schauenstein, and Sekora 1950).

The contents of the gland are too concentrated to provide material suitable for electron microscopy. It was therefore necessary to explore the possibility of using dilute solutions. Various earlier workers, including Foa (1912), Ludwig, and Dubois (see Bergmann 1939), found that the gland contents were partly soluble in water. According to these workers it is the precursor of fibroin (fibroinogen) alone that is soluble in water—the sericin precursor being insoluble. The correctness of this observation was assumed in the present work, but needs confirmation. The aqueous solution of fibroinogen behaves similarly to solutions of other proteins and this justifies the conclusion that insoluble fibrous fibroin is developed from a non-fibrous precursor and that the transformation may bear a resemblance to that of other fibre-forming systems better studied, e.g. G- and F-actin, fibrinogen-fibrin. Unfortunately, there is no information available concerning the molecular characteristics of fibroinogen, but it is hoped to obtain this shortly.

Solutions of fibroinogen freshly prepared have a slight turbidity, which increases in the course of a few hours. After a variable length of time (4-12

hours) the protein separates as a flocculent precipitate. This phenomenon was studied with the electron microscope.

Silk glands were removed from fully grown caterpillars of *B. mori,* washed briefly in distilled water, and placed in distilled water at room temperature. The gland membrane was gently slit to allow the contents to enter solution and the whole stood for about one hour without disturbance. At the end of this time the extract was gently stirred and the undissolved material centrifuged off. The extract was faintly turbid and contained approximately 0.1 per cent. of dissolved protein. The observations previously reported by Foa (1912) were confirmed. Drops of this solution and that of a further solution diluted to 1/10th strength were dried directly on collodion films, shadowed with uranium, and examined in the microscope. Such specimens revealed only indeterminate clumps of material, no fibrous and no well-defined particulate forms.

On standing, the turbidity of the extract of the gland increased and in some hours (9-24) a precipitate began to separate. After centrifugation and washing the precipitate was X-rayed and was found to yield a pattern similar to unoriented silk fibre. Drops of the turbid extract were dried on collodion films and shadowed. Examination in the electron microscope revealed clumps of fibrils such as shown in Plate 1. The turbidity is thus associated with the spontaneous development of these fibrils, which become entangled and separate as a precipitate.

On comparing electron micrographs such as Plates 1 and 2 with micrographs of actin and fibrin, one notes in the silk derivatives the absence of a tendency for the fine fibrils to clump together, e.g. as tactoids, or to build larger fibrils, which is so obvious a characteristic of the other two fibre-forming proteins. Whether this feature is related to the absence of macrofibrils in silk fibre is an interesting question.

The observation that fibroinogen in dilute solution forms microfibrils spontaneously is not direct evidence that naturally formed fibroin possesses a fine structure, since it involves the assumption that the fibrillation in dilute solution is similar to that which occurs in the concentrated solution of the actual gland. The problem needs fuller investigation and the present evidence is only suggestive. A difference in appearance — particularly length — between the microfibrils of silk fibre and these of Plate 2 is obvious. This does not affect the conclusion that fibroinogen possesses the property of forming fibrils by what appears to be a simple aggregation in the absence of mechanical orienting influences, a property displayed by other fibre-forming substances and suspected of being a fundamental step in the formation of biological fibres.

(*c*) *Fine Structure of Regenerated Silk Fibroin*

Coleman and Howitt (1947), whose valuable work redirected interest towards this fibre as a useful substance for the study of fibre formation, prepared soluble derivatives by dispersing the fibre in cupriethylene diamine solution. They called their soluble silk "fibroinogen" and assumed its identity with, or

close similarity to, the soluble precursor of the silk gland, to which this name is more correctly applied. However, the molecular characteristics of their soluble derivatives cannot be ascribed with certainty to the silk gland proteins until a further comparison of the two materials is made. Silk fibre regenerated from the solutions of Coleman and Howitt gives an X-ray pattern similar to the original silk. This also does not suffice to establish identity since a variety of break-down products of sufficiently high molecular weight could be expected to yield the same large-angle pattern as is also the case with solutions of wool (Mercer 1949), feather (Lundgren 1949), or cellulose (Meyer 1942).

Since soluble silk can be readily induced to revert to the insoluble form it was decided to examine such regenerated fibre for the presence of fine structure.

Silk fibroin prepared as in Section II(*a*) was dispersed in 50/50 cupriethylene diamine as described by Coleman and Howitt (1947). The solution was dialysed until free from copper, and fibrous products prepared in two ways:

(i) By allowing the solution to stand until a surface scum of insoluble regenerated fibroin appeared.

This was scraped off, washed, and dispersed into fine fragments in the Waring Blendor. Examination in the light microscope and in the electron microscope showed that the fragments formed thin sheets which did not seem to be mats of a fibrillar texture but were more homogeneous.

(ii) By diluting the solution to about 0.5 per cent. protein and allowing it to stand until it became turbid and set to a weak gel. This gel was then dispersed in water and examined with the electron microscope. In this case the fragments were found to be particulate and of no ascertainable structure.

III. Discussion

At the present time great interest is being displayed in the problem of the formation of fibres in biological systems. In most cases it is thought that a non-fibrous percursor is at first synthesized and that this is subsequently transformed into the fibrous form. Silk formation obviously offers a good example of this change. Theories to account for the transformation (often referred to as the G-F transformation (Wyckoff 1949)) may belong to one of two classes: (i) *Aggregation theories* according to which the precursor is a more or less symmetrical particle with the property of aggregating to long strings (or more complex formations) forming the protofibrils of the fibre. (ii) *Chain-unfolding theories* in which the precursor molecule unfolds into a long chain, extends, and crystallizes with other chains to form long micelles of the "fringed" variety (Meyer 1942). In the protein fibres the chain-unfolding hypothesis is often stated to be a type of extreme "denaturation." Under natural conditions, there is no convincing evidence of denaturation, but it may occur in the process of making fibres artificially.

Experimental evidence to distinguish between the implications of the two theories is not easy to come by since, as far as most properties of the fibres are concerned, both theories explain the facts equally well. Instances in natural systems, in which a seemingly slight, even reversible, change precipitates the appearance of fibrils, are not easily explained by denaturation and seem to point rather to aggregation. It is at the level of fine structure, however, where the two theories, interpreted in their simplest forms, predict different structures. The most probable fine structural feature to be expected from the crystallization of long chains is the "fringed micelle"; on the other hand linear aggregation might be expected to produce long, discrete fibrils of constant diameter. The fringed micelle was proposed to reconcile the micellar structure, implied by X-ray studies, with the co-existence of chains longer than the micelles themselves. Only since the development of the electron microscope has the existence of long microfibrils of constant diameter been established and given support to aggregation theories.

On the grounds of its established molecular structure (Meyer 1942) and the way in which the silk-worm spins the fibre, silk fibroin would seem to be a clear example of a fibrous form produced by the unfolding of molecular chains. However, the present work shows that silk, in common with other protein fibres, possesses a fine structure of microfibrils and that moreover fibroinogen, the soluble precursor of the insoluble fibre, displays the property of forming fibres spontaneously without the participation of mechanical forces. These findings emphasize that silk has greater structural similarity to other natural protein fibres than to artificial fibres produced by extrusion and drawing. The activities of the silk-worm during spinning are thus not entirely analogous to those occurring in artificial spinning.

Assuming the existence of microfibrils and their formation by aggregation a possible sequence of events during the natural spinning process would be as follows:

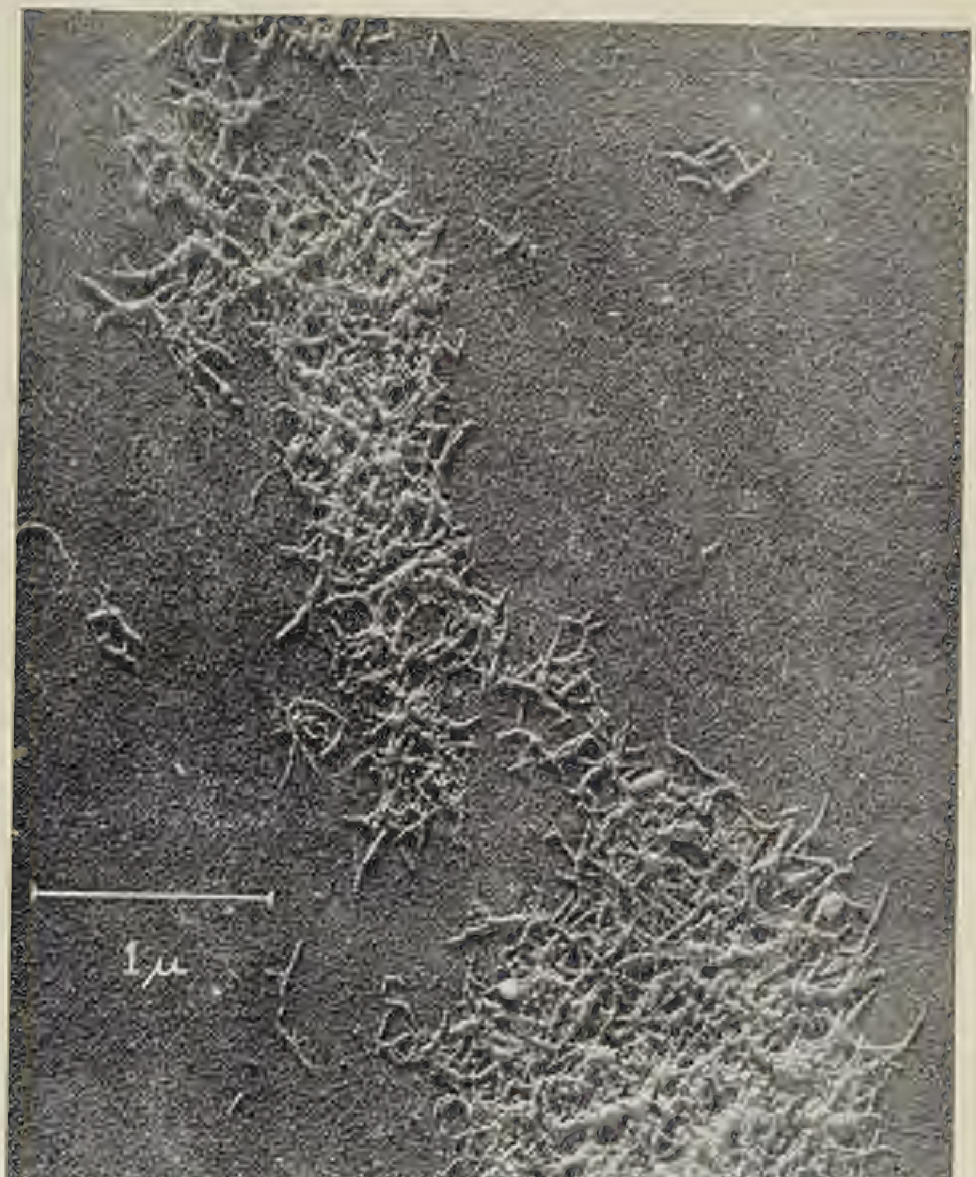
(i) Syntheses of soluble precursor (G-form) — fibroinogen (primary synthesis);

(ii) The aggregation of the precursor to give microfibrils (protofibrils), which may be called *fibrillation*;

(iii) Orientation of the mass of microfibrils by the viscous shear produced during the passage of the gland contents through the silk press and subsequent extension.

The difference between this theory and the accepted scheme lies in the absence of molecular chain unfolding produced by stretching. It is assumed that the precursor molecules are incorporated intact into the protofibrils. Clearly, in order to produce the molecular orientation revealed by X-rays, it must be further assumed that these molecules are oriented on aggregating to form the fibril.

Fibrillar fragment from silk subjected to prolonged digestion with trypsin. Note fine, parallel fibrils. Shadowed with uranium.



Fine fibrils appearing spontaneously in dilute solutions of fibroinogen obtained from the silk gland. Shadowed with uranium.

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SPECIFIC DYNAMIC ACTION OF ACETIC ACID AND HEAT INCREMENT OF FEEDING IN RUMINANTS

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Summary

Attention is drawn to the lack of adequate explanation of the phenomena of the high specific dynamic action of acetic acid and the high heat increment of feeding in ruminants.

The theory is advanced that the high specific dynamic action of acetic acid is related to the following facts: it is non-glyconeogenic; it is not utilized in protein synthesis; it is oxidized by most, if not all, tissues; and it is utilized significantly for lipogenesis by only a few tissues, excluding in particular muscle and kidney. Furthermore, the metabolism of the acid appears to be relatively "uncontrolled," the uptake by tissues being directly dependent on the arterial level and unaffected by insulin, at least in ruminants, in contrast to glucose.

Finally, there is very little storage of absorbed acetic acid in the body fluids. Consequently, it is metabolized almost as fast as it is absorbed: in some tissues, notably intestinal wall, liver, and adipose tissue and lung, it is partitioned between oxidation and lipogenesis; in others, particularly muscle and kidney, it is of necessity largely utilized oxidatively. The high specific dynamic action of acetic acid indicates that the net partition is in favour of oxidation.

The high heat increment of feeding in ruminants is considered as due to the quantitative importance of acetic acid and butyric acid, which also has a high specific dynamic action, as products of ruminal digestion, and of acetic acid and β-hydroxybutyric acid, derived from acetic and butyric acids, as peripheral metabolites.

I. Introduction

Two long-recognized phenomena, the high specific dynamic action of acetic acid and the high heat increment of feeding in ruminants have as yet failed to receive any adequate interpretation or explanation.†

The high S.D.A. of acetic acid was first demonstrated by Lusk in 1921 when it was shown that, per unit weight of material, the increase in the metabolic rate of a dog following feeding of acetic acid was very considerably greater than that following feeding of glucose; 3 g. of acetic acid increased the resting metabolic rate by 3.1 cal./hr., and 50 g. of glucose by 4.7 cal./hr. The principle of this observation has been several times confirmed. In nephrectomized dogs, the average excess oxygen consumption following intravenous

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† "Specific dynamic action" and "heat increment" are usually regarded as synonymous but in this paper "specific dynamic action" or "S.D.A." will be used in referring to the effect of specific nutrients or metabolites in increasing metabolism and "heat increment of feeding" to the post-feeding increase in metabolism that follows ingestion of food in general.

injection of sodium acetate was found to be 49 per cent. of that required for complete oxidation of the acetate (Dye and Masters 1944). In the sheep Marston has been reported (Barcroft 1947) as recording a specific dynamic action of 70 per cent. for acetic acid, as against 30 per cent. for the glycogenic propionic acid. In man, continuous intravenous infusion of sodium acetate resulted in the same rate of increase of serum carbon dioxide as did administration of an equivalent amount of sodium bicarbonate (Mudge, Manning, and Gilman 1949), suggesting a very rapid oxidation of the acetate.

The high heat increment of feeding in ruminants, amounting to 30-60 per cent. of the metabolizable energy of the food (gross energy minus energy lost in faeces, urine, and methane) was first described by Armsby and Fries in 1903, and has been repeatedly confirmed (Ritzman and Benedict 1938; Forbes, Braman, and Kriss 1928, 1930; Marston 1949). The findings that acetic acid is a major product of ruminal digestion (Barcroft, McAnally, and Phillipson 1944; Elsden *et al.* 1946; Marston 1948; Kiddle, Marshall, and Phillipson 1951; McClymont 1951*a*), that on a roughage diet virtually no enzymically digestible carbohydrate escapes ruminal fermentation to the volatile fatty acids (Heald 1951; McClymont 1949), and that acetic acid is a major tissue metabolite of ruminants (Reid 1950; McClymont 1951*b*), strongly suggest that the high heat increment of feeding of ruminants may be largely attributable to the high S.D.A. of the acetic acid produced in ruminal fermentation. It has been shown by Marston (1948), by experiments *in vitro*, that the actual heat of fermentation in the rumen could probably account for not more that 15 per cent. of the heat increment. However, despite the importance of acetic acid and heat increment of feeding in ruminant physiology and nutrition, no theory providing any fundamental interpretation or explanation of the phenomena has, as yet, been put forward, although the probable relation of the heat increment to metabolism of the volatile fatty acids produced in the rumen has been suggested by Ritzman and Benedict (1938) and Marston (1948). Also, Reid (1950) has made the significant suggestion that "when fermentation in the rumen is intense, acetate might be absorbed at such a rate that much of it would be oxidized without contributing useful energy to the animal." The picture now emerging of the pathways by which acetic acid and glucose are metabolized and of the metabolism of the volatile fatty acids by ruminants, is considered to provide sufficient evidence on which such a theory may be based.

Firstly a theory is presented concerning the specific dynamic action of acetic acid and then this theory is incorporated in a consideration of the heat increment of feeding of ruminants.

II. Theory Relating to the Specific Dynamic Action of Acetic Acid

The evidence and arguments involved in this theory are presented hereunder, and summarized in diagrammatic form in Figure 1.

(*a*) *Acetic acid is non-glyconeogenic in animal tissues.* This has been evidenced by a diversity of experiments, including use of phloridzinized dogs (Ringer and Lusk 1910; Deuel and Milhorat 1928) and sheep (Jarrett and Potter 1950), liver glycogen formation experiments (Deuel *et al.* 1937), and failure of acetic acid, but not propionic acid, to affect the insulin coma or hypoglycaemia of sheep (Reid 1951). The appearance of labelled carbon from acetate in all carbon atoms of glucose can be readily explained by the tricarboxylic acid cycle theory without assuming glyconeogenesis (Potter and Heidelberger 1950). Nor is acetic acid utilized as a building block in formation of body proteins (Potter and Heidelberger 1950; Greenberg and Winnick 1949).

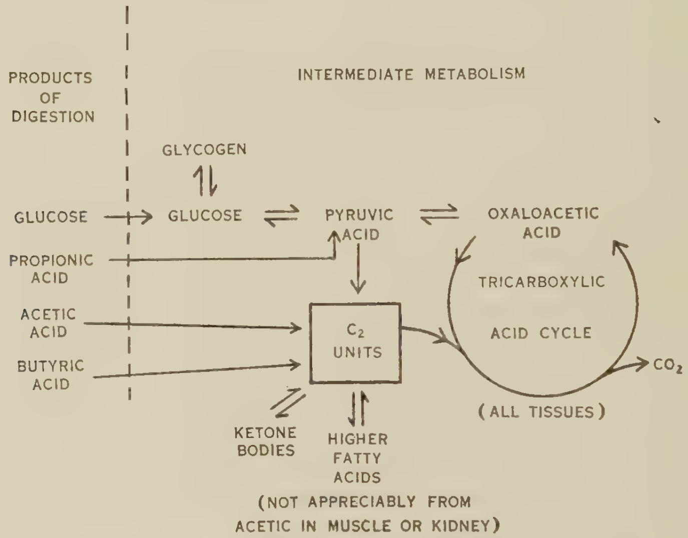

Fig. 1.—The major alternative metabolic pathways available to fatty acids and to glucose. In addition glucose may be utilized in synthesis of the non-essential amino acids.

The irreversibility of the pyruvic acid $\longrightarrow$ C_2 unit step is considered to be the key factor determining the wide difference between the metabolism of glucose and of acetic acid, and the restriction of the C_2 unit $\longrightarrow$ higher fatty acid step to certain tissues a major factor in the high S.D.A. of acetic acid.

(*b*) *Acetic acid is oxidized by many,* possibly all, individual tissues, including heart muscle (Lorber *et al.* 1946; Pearson, Hastings, and Bunting 1949; Pearson *et al.* 1949), diaphragm muscle (Pearson *et al.* 1949), gastrocnemus muscle (Lifson, Omachi, and Cavert 1951), lung, liver, and kidney (Pardie, Heidelberger, and Potter 1950), and mammary gland (Folley and French 1950; Popják *et al.* 1951). Brain has been reported not to utilize acetic acid *in vitro* (Long and Peters 1939) but it is possible that the finding may be due to initial exhaustion of the tricarboxylic acid cycle by which acetic acid is oxidized.

(*c*) *Acetic acid is utilized for fat synthesis.* This is so in the intact body and liver of the rat (Bloch 1947), in the mammary gland of ruminants (Folley and French 1950; Popják *et al.* 1951), in the liver, intestinal wall, and lung of the rabbit and lung of the ruminant (Popják and Beeckmans 1950), and in adipose tissue of the rat (Hangaard and Marsh 1952); but it is not detectably utilized for this purpose by at least heart and diaphragm muscle (Pearson, Hastings, and Bunting 1949; Pearson *et al.* 1949) or kidney of the rat (Pardie, Heidelberger, and Potter 1950; Elliott and Kalnitsky 1950), the kidney experiments showing that practically all acetate metabolized was oxidized. It is also utilized in synthesis of other minor tissue constituents, notably cholesterol and porphyrins (Bloch 1947). It has also been shown that with glucose as substrate the ratio of the conversion *in vitro* of glucose to fatty acids and to carbon dioxide was 1 : 6 in liver and 1 : 8 in muscle (Chernick, Masoro, and Chaikoff 1950). This coupled with evidence of a higher rate of turnover of fatty acids in liver than in muscle (Chernick, Masoro, and Chaikoff 1950), is evidence of a far greater tendency for oxidation than lipogenesis in muscle. The ability of the peripheral tissues, largely muscle, to metabolize acetic acid is well demonstrated by the rapid metabolism of the acid in eviscerated nephrectomized dogs (Dye and Masters 1944).

(*d*) *On absorption acetic acid does not accumulate in the tissues;* and of course, could not be accumulated to any extent without precipitating an acidosis, the maximum content of the blood during the height of ruminal fermentation usually being only about 15 mg. per cent. (Reid 1950; McClymont 1951*b*). That is, acetic acid is metabolized practically as fast as it is absorbed. The extremely high arterio-venous differences of acetic acid in ruminants, of the order of 20 per cent. of the arterial level in the head of the sheep (Reid 1950) and 40-80 per cent. in the lactating bovine mammary gland (McClymont 1951*b*), are reflections of this rapid metabolism.

To summarize, the evidence shows that acetic acid is oxidized by many, possibly all, tissues, but is utilized to a significant extent for lipogenesis by only a small proportion of them, since the muscles, which constitute the largest mass of the body, and the kidney, which is another very metabolically active tissue, apparently are not included in this category; and it is not available in any tissue for glyconeogenesis or protein synthesis. The evidence also indicates that acetic acid absorbed from the gastro-intestinal tract will be successively partitioned between oxidation and lipogenesis by the intestinal wall (at least in the rabbit; there is as yet no evidence of lipogenesis in the intestinal wall of the ruminant), by the liver, and by the lung; then on reaching the general circulation it will be distributed in equal concentration to all tissues, some of which, namely intestinal wall, liver, lung, mammary gland, and adipose tissues, are capable of using it for both lipogenesis and oxidation. But a large proportion of the tissues, at least skeletal and cardiac muscle and kidney, will be capable of metabolizing it to any significant extent only by oxidation. Even in a tissue where acetic acid is lipogenic, as the lactating ruminant mammary gland (Folley and French 1950), it appears that only a small proportion of

the acetic acid utilized is actually used for lipogenesis, the remainder being necessarily oxidized (McClymont 1951*b*).

It is considered that this rapid metabolism of acetic acid, with partition between oxidation and lipogenesis in lipogenic tissues, and necessarily oxidative metabolism by non-lipogenic tissues, causes a high proportion of all acetic acid to be rapidly oxidized, and that this accounts largely for the high specific dynamic action of acetic acid.

Discussion of S.D.A. Theory

It is also probable that the specific dynamic action values recorded for acetic acid are underestimates of the amount actually oxidized, since there is an inverse relationship between glucose and acetic acid uptakes, at least by the head tissues of the sheep (Reid 1950): a rise of 2 mg. per cent. in arterio-venous difference of acetic acid depressed glucose uptake by approximately 1 mg. per cent. Competitive depression of metabolism of two-carbon-atom units from glucose by acetic acid might be involved in this example, but a more complex and profound relationship between acetate and glucose metabolism is indicated by the specific effect of acetate in depressing glucose oxidation and stimulating glycogenesis in rat tissues *in vitro* and *in vivo* (Parnas and Wertheimer 1950). However, in the dog the specific dynamic actions of acetic acid and glucose were reported by Lusk (1921) to be independent.

The fundamental divergence in the metabolism of glucose and acetic acid, leading to the marked difference in the efficiency of utilization of energy from the two metabolites, is considered to be that ingested glucose may be stored as glycogen in practically all tissues pending its disposition to oxidation, lipogenesis, synthesis of non-essential amino acids, or formation of oxaloacetic acid for the tricarboxylic acid cycle. Such storage is also at very little energy cost (Baldwin 1947) and pending such storage glucose may be built up to considerable levels in the body fluids without ill effect. Even adipose tissue, under the influence of a sudden large glucose intake or of hyperinsulinism, may store appreciable quantities of glycogen that is later utilized for lipogenesis (Wertheimer and Shapiro 1948). On the other hand, since acetic acid is non-glycogenic and thus cannot be temporarily stored as glycogen, and since it is not stored in the blood, it must, unlike glucose, always be rapidly partitioned between oxidation and lipogenesis.

It would seem that the rapid rate of oxidation of acetic acid by the tricarboxylic acid cycle, resulting as it does in extremely inefficient utilization of the energy liberated, is evidence of a relatively "automatic" or "uncontrolled" oxidation of the acid by the cycle in non-lipogenic tissues. This is in marked contrast to glucose, which is subjected to a multiplicity of enzymic and, until the last step, reversible, degradations to a two-carbon-atom unit, which is then oxidized by the tricarboxylic acid cycle or utilized for fatty acid synthesis.

As acetic acid appears to be the stabilized form of the two-carbon-atom units from pyruvic acid (Coxon 1950) and fatty acids (Bloch 1947) which enter the tricarboxylic acid cycle, it would seem that the metabolic disposal of C_2 units in non-lipogenic tissues, being a final step, is not very delicately

controlled. Such a relatively "uncontrolled" metabolism of the C_2 unit acetic acid, in comparison with glucose, is evidenced by the straight-line relationships between arterial levels and arterio-venous differences of acetic acid in the head of the sheep (Reid 1950) and bovine mammary gland (McClymont 1951*b*), suggesting a simple mass-action effect, and by the high percentage arterio-venous differences in ruminants, which are not affected, *in vivo*, by hyperinsulinism (Reid 1949; McClymont 1951*b*). Furthermore *in vitro*, insulin does not stimulate lipogenesis from acetate in ruminants although there is some evidence that it may do so in non-ruminants (Balmain and Folley 1951). In contrast, at least in man, arterio-venous differences of glucose are far from directly dependent on the arterial level and are greatly modified by hyperinsulinism and the resultant "opposing factors" (Somogyi 1948, 1949).

Another consideration is that the condensation of glucose or acetic acid to higher fatty acids, being an endergonic reaction, must necessitate an exothermic oxidation of substrate to provide the necessary energy: the energy transfer cannot be completely efficient, as has been discussed by Borsook and Winegarden (1930). There is evidence that when carbohydrate is metabolized under conditions where it must be largely used for lipogenesis, i.e., following massive ingestion or infusion of carbohydrate, the increase in heat production is equivalent to the oxidation of 20 per cent. or more of the glucose metabolized (Rapport, Weiss, and Csonka 1924; Wierzuchowski and Ling 1925; Wierzuchowski 1937). If this figure is applicable to lipogenesis from acetic acid, which has nearly the same combustible energy per gram and the same empirical formula as glucose, and if there is not sufficient "uncontrolled" oxidation of acetic acid in lipogenic tissues to provide the energy required for the condensation of other acetic acid molecules, then this factor also could explain an appreciable proportion of the S.D.A. of acetic acid.

An experimental finding that requires reconciling with the present theory is that where tri-acetin replaced 15 per cent. of glucose in a rat diet on an equicaloric basis, paired feeding experiments over 7 months gave equal results as regards growth and metabolic rates (McManus, Bender, and Garrett 1943), an unexpected finding in view of the high S.D.A. of acetic acid reported by others. However, this result can be reconciled if it is assumed that the food was eaten and the acetic acid hydrolysed from the tri-acetin at such a rate that the acetic acid could join the pool of endogenous "acetic acid" in the liver, which undergoes a rapid turnover (Bloch 1947), without raising the peripheral blood level of acetic acid to any appreciable extent. Without this raised blood level the necessarily oxidative utilization of acetic acid by muscle and kidney tissue would not occur and the net energy value of calories from glucose and acetic acid could be approximately equal.

III. Theory Relating to the Heat Increment of Feeding in Ruminants

Turning now the problem of the high heat increment of feeding in ruminants, it is considered in the light of evidence cited above that this heat increment is attributable in a very large degree to the high S.D.A. of the acetic

acid produced during ruminal digestion and carried to all tissues in the arterial blood.

However, butyric acid, another major product of ruminal digestion, and a fatty acid with an even number of carbon atoms, will also be metabolized as C_2 units. These will follow, perhaps in somewhat different proportions because of the two types of C_2 units, acetyl and carboxy-methyl, from butyric acid (Potter and Heidelberger 1950; Kennedy and Lehringer 1950), the same metabolic pathways as acetic acid and will thus also contribute to the heat increment of feeding. The high S.D.A. (Dye and Masters 1944), the non-glycogenicity (Deuel *et al.* 1937), and the lipogenicity (Brady and Gurin 1950) of butyric acid have all been demonstrated. That much of the butyric acid and some acetic acid is probably converted in the rumen wall (Pennington 1951) and the liver to ketone bodies, largely β-hydroxybutyric acid, which are then metabolized by the peripheral tissues (Shaw and Knodt 1941; McClymont 1949), would not affect the fate of rapid partition between lipogenesis and oxidation. Ketone bodies are metabolized, in at least the fed bovine, at a rate dependent on the arterial level (McClymont 1949), as is acetic acid, and are non-glycogenic (Stadie 1945), which is only to be expected, since they are products of the non-glycogenic acetic acid.

Other minor products of ruminal fermentation such as valeric and hexanoic acids (McClymont 1951*a*; Gray *et al.* 1951) will contribute three or nil carbon atoms for glyconeogenesis, depending on whether they contain an odd or even number of carbon atoms, and the remaining atoms will follow the pathways of acetic and butyric acids.

There is some dispute as to the actual proportions of the volatile fatty acids produced in ruminal digestion (Gray and Pilgrim 1950; Kiddle, Marshall, and Phillipson 1951).

Discussion of Heat Increment Theory

It is intriguing that acetic acid, such a major product of digestion in ruminants, is so inefficiently utilized. It would seem that although plants, e.g. developing oil seeds, can convert fats to carbohydrates and microorganisms can utilize acetic acid as a source of carbohydrate, mammalian (and perhaps all animal) tissues evolved without the enzyme systems capable of converting two-carbon-atom compounds to glucose precursors. The ability of bacteria to carry out carbohydrate synthesis from acetic acid depends apparently on enzyme systems catalysing the condensation of two molecules of acetic to the glycogenic succinic acid (Barron, Ardao, and Hearon 1950). Condensation of formate and acetate to pyruvate has not yet been proven (Strecker 1951). It appears that the only enzyme systems involved in quantitatively significant metabolism of two-carbon-atom units in animal tissues are those involved in the metabolism of such compounds from fatty acids and from pyruvic acid, in oxidation, in acetylation, in synthesis of fatty acids and of some other minor body constituents such as cholesterol, and in metabolism of glycine.

The evolution of ruminants has led to many changes that can be interpreted as adaptations to the radical change in the nature of digestion products occasioned by the circumstance that all feed is first exposed to bacterial digestion. These include low blood glucose, reduced sensitivity to insulin, and small glucose arterio-venous differences (Reid 1951). However, it has apparently failed to lead to an enzymic adaptation permitting glyconeogenesis from acetic acid. If this had occurred there would have been, if the present theory is correct, a considerable increase in the ruminants' low efficiency of utilization of metabolizable energy. It is interesting to speculate upon the effects this might have had on the evolution and characteristics of the ruminant.

Glyconeogenesis from propionic acid, the other major product of ruminal digestion, appears to be due to an enzyme, reported so far in rabbit liver but not in kidney (Huennekens, Mahler, and Nordmann 1951), which may be the result of enzymic adaptation to bacterial digestion. It will be of interest to learn how widely this enzyme is distributed among species with varying degrees of dependence on bacterial digestion. Possibly the enzyme is an adaptation, to the special case of the three-carbon-atom fatty acid, of the enzyme responsible for the normal oxidation of fatty acids, and glyconeogenesis may be only incidental to β oxidation of propionic acid, leading via acrylic acid to the glycogenic lactic acid (Huennekens, Mahler, and Nordmann 1951).

It is recognized that some key assumptions have been made in the above arguments. For example, it has been assumed that the finding of lack of appreciable fat synthesis by rat muscle *in vitro* is applicable to ruminant muscle *in vivo,* and that the finding of the non-glycogenicity of butyric acid in rats is true for ruminants. Experimental confirmation of these points and further data on the lipogenicity of various tissues would assist considerably in affirming or denying the validity of the present theory.

The theory suggests some intriguing problems in ruminant physiology. Forbes, Braman, and Kriss (1928, 1930) have, for example, concluded that the net energy of feeds decreases with increasing food intake. Does the rate of ruminal fermentation of food, which may be affected by amount fed, rate of feeding, and physical condition of the feed, influence, through an effect on blood levels of acetic acid, the proportions of the acid wastefully oxidized and used for fat synthesis, or alternatively does it influence the proportions of the glycogenic propionic acid, and the non-glycogenic acetic and butyric acids, and thus influence the net energy value of feed? Or does Marston's (1949) finding of a constant proportion of metabolizable energy lost as heat increment indicate that a constant proportion of acetic acid is always used for lipogenesis?

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THE NATURE OF REACTION WOOD

III. CELL DIVISION AND CELL WALL FORMATION IN CONIFER STEMS

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Summary

Cell division, the nature of extra-cambial readjustment, and the development of the secondary wall in the tracheids of conifer stems have been investigated in both compression wood and normal wood. It has been shown that the reduction in tracheid length, accompanying the development of compression wood and, in normal wood, increased radial growth after suppression, result from an increase in the number of anticlinal divisions in the cambium. From observations of bifurcated and otherwise distorted cell tips in mature tracheids, of small but distinct terminal canals connecting the lumen to the primary wall in the tips of mature tracheids, and of the presence of only primary wall at the tips of partly differentiated tracheids, and from the failure to observe remnants of the parent primary walls at the ends of differentiating tracheids, it has been concluded that extra-cambial readjustment of developing cells proceeds by tip or intrusive growth. It has been further concluded that the development of the secondary wall is progressive towards the cell tips, on the bases of direct observation of secondary wall formation in developing tracheids and of the increase found in the number of turns of the micellar helix per cell with increasing cell length. The significance of this in relation to the submicroscopic organization of the cell wall has been discussed. Results of X-ray examinations and of measurements of tracheid length in successive narrow tangential zones from the cambium into the xylem have indicated that secondary wall formation begins before the dimensional changes of differentiation are complete.

I. INTRODUCTION

The development of compression wood in conifer stems is characterized by a shorter average tracheid length, in comparison with that of previously formed normal wood, and by readily recognizable morphological features such as the rounded form of the tracheids, the intercellular spaces, and the helical checking in the secondary wall. In the second paper of this series (Wardrop and Dadswell 1950) it was suggested that the reduction in tracheid length resulted from an increase in the number of anticlinal divisions in the cambium associated with the rapid eccentric radial growth (periclinal divisions) that is usually associated with the development of compression wood.

In conifer stems generally the importance of anticlinal divisions in accommodating the increased girth of the stem was recognized by Bailey (1923) and by Priestley (1930). However, the recent work of Bannan and Whalley (1950) has demonstrated that the frequency of these divisions is very much greater than was previously supposed. From the changing pattern of the differentiating

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tracheids seen in longitudinal tangential sections, these investigators have concluded that increase in length of the daughter cells, following anticlinal divisions, results from tip growth. The development of the secondary wall is known to begin first in the region of the pits, but whether it continues to develop simultaneously over the entire inner surface of the primary wall does not appear to be known. Compression wood is particularly suitable for the further investigation of these problems. The rapid radial growth and the sudden reduction in tracheid length provide material in the formation of which, if the above assumption is correct, anticlinal divisions are frequent and the nature of the increase in length of the daughter cells of such divisions can be observed.

It has been shown previously (see Wardrop and Dadswell 1950) that the secondary wall of compression wood tracheids consists of two layers, the outer layer in which the micellar angle is large (78-90°) in relation to the longitudinal cell axis and the inner wider layer in which the micellar angle is smaller (30-45°). The helical checks peculiar to the inner layer provide a basis for analysing the relation between fine structure and cell wall development more exactly than can be done with normal wood cells. Preston (1934, 1948) showed that cell length in conifer stems was linearly related to the cotangent of the angle between the direction of the cellulose micelles in the helically organized middle layer of the secondary wall and the longitudinal cell axis. This relation has been shown to apply to all layers of the secondary wall of normal tracheids (Preston and Wardrop 1949; Wardrop 1952*a*) and implies that the number of turns of the micellar helix per cell is constant. However, in a study of the orientation of helical thickenings in the tracheids of *Pseudotsuga* and *Taxus*, where the inclination to the cell axis also decreases with increasing cell length, it was shown (Wardrop and Dadswell 1951*a*) that the number of turns of the helical thickening increased with increasing cell length. It was considered that this might indicate a directed synthesis of cellulose in the thickenings but, before such a consideration can be accepted for cell wall development generally, it is necessary to obtain cells in which the number of turns of the micellar helix can be measured. Such cells are provided by compression wood, in which the length-micellar angle relationship of Preston is known to hold (Wardrop and Dadswell 1950).

In the present investigations consideration has been given to the frequency of anticlinal divisions in the cambium, to the nature of the readjustment of the daughter cells, and to the development of the secondary wall during differentiation of compression wood tracheids. Because of the general implications of these questions parallel studies have, as far as possible, been carried out with normal wood tracheids.

II. Experimental

(*a*) Cell Division in the Cambium

Two specimens of *Pinus pinaster* Sol. were selected for examination. In specimen 1, of unknown age, both normal wood and severe compression wood were

present and growth rings were not discernible in the compression wood zone. The formation of compression wood in the specimen had involved a rapid in-

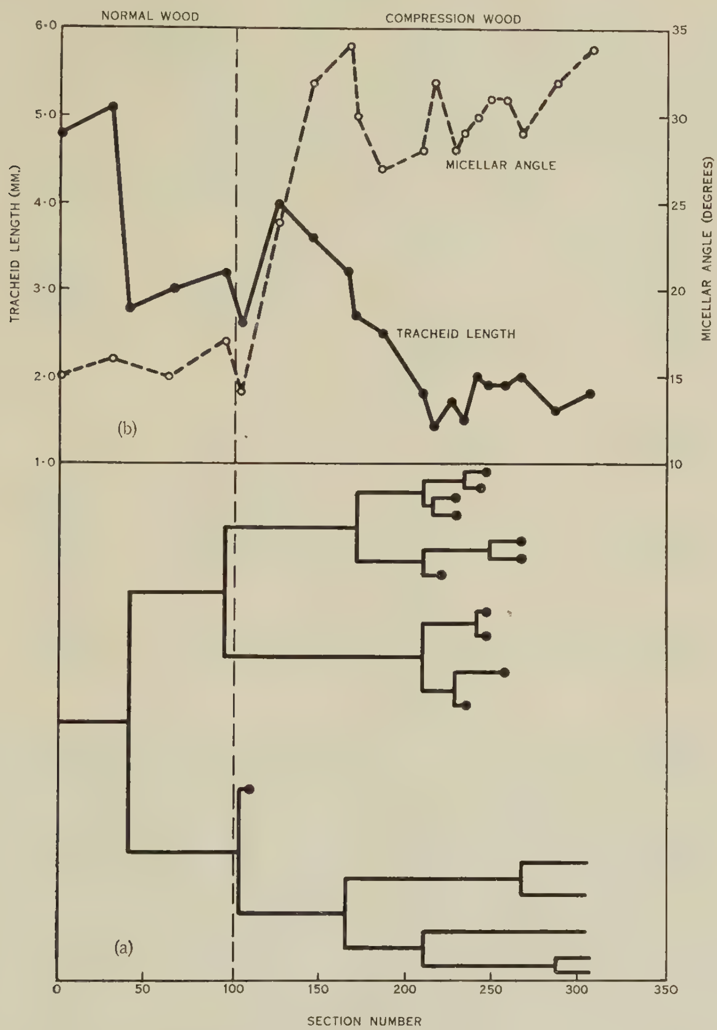

Fig. 1.—(a) Sequence of anticlinal divisions in a single radial file of tracheids as observed in successive tangential sections extending through a zone of developing compression wood. The black dots represent the elimination of a file of tracheids. The scale of the abscissa is the section number from the initial point of observation. The vertical broken line indicates the point at which features of incipient compression wood were first observed. (b) Tracheid length (mm.) and micellar angle (°) as measured in the series of tangential sections depicted in (a).

crease in radial growth rate and was accompanied by considerable decrease in tracheid length. Specimen 2 was taken from a concentric stem showing 31

growth rings. The growth of the tree had been retarded and had responded to treatment with superphosphate resulting in a considerable increase in radial growth rate after the 14th ring, with decrease in tracheid length, but without the formation of compression wood. Serial longitudinal tangential sections, 30 μ thick, were cut from specimen 1 over the region of initial compression wood formation and from specimen 2 over growth rings 14 and 15, where there was the sudden increase in radial growth rate. Each section, as cut, was numbered according to the growth ring and its position in the ring, or, where no growth rings were evident, according to its position in the specimen. All

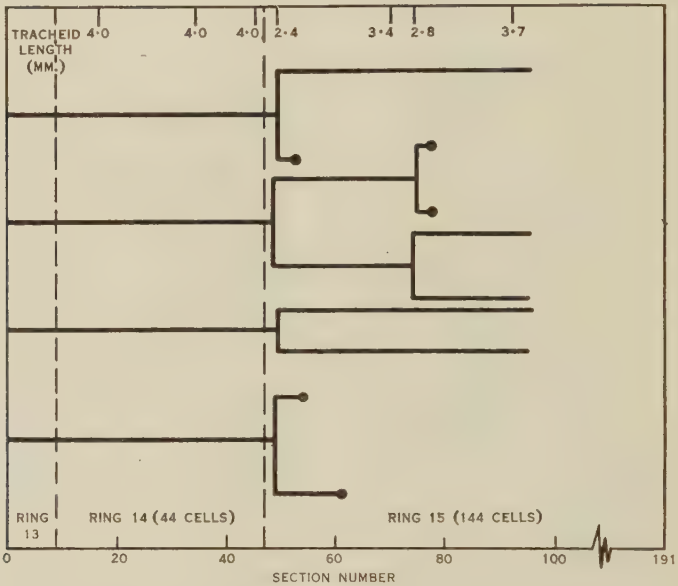

Fig. 2.—Sequence of anticlinal divisions in four radial files of tracheids followed through three growth rings of a specimen of *Pinus pinaster*. The scale on the abscissa as in Figure 1.

sections were examined microscopically to determine the sequence of anticlinal divisions according to the procedure adopted by Bannan and Whalley (1950). This method, used originally by Klinken (1914), involves a number of assumptions, among the most important of which are:

(i) The arrangement and length of the tracheids approximately recapitulate the arrangement and length of the fusiform initials of the cambium at the time the tracheids were cut off;

(ii) The appearance of two tracheids as seen in serial tangential sections, instead of one, has involved the occurrence of an anticlinal division; and

(iii) The disappearance of a tracheid from a radial file has involved the disappearance of a fusiform initial from the cambium.

Some support for the first of these assumptions may be found in the observations of Bailey (1920) that tracheids of conifers are only 5-20 per cent. longer

than the cambium initials from which they are derived. The other assumptions, although justifiable, may be complicated by other factors such as the fact that cell division is not confined to a single initial or tangential row of initials—the cambium in Sanio's sense—but involves a radial zone of cells as proposed by Raatz, Schoute (see Meeuse 1941), and Wight (1933).

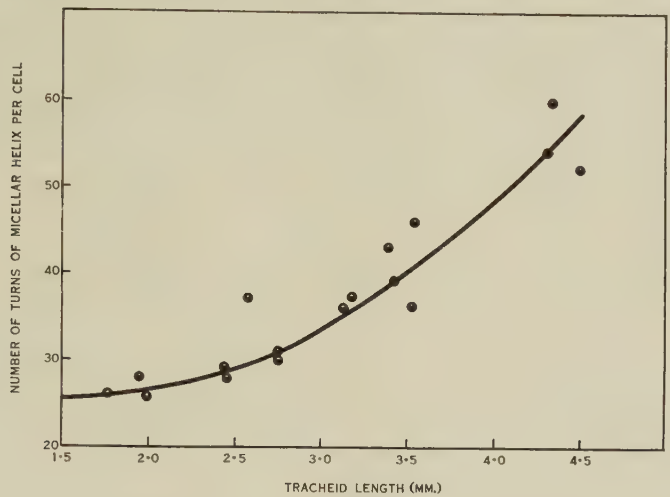

Fig. 3.—Variation in the number of turns of the micellar helix per tracheid with tracheid length in compression wood of *Pinus pinaster.*

The results obtained in the present investigation, on the sequence of divisions for selected radial files of cells, are shown in Figure 1 for specimen 1, and in Figure 2 for specimen 2. The position in the specimen is indicated by the section numbers. In specimen 1 one original radial file was followed through 306 sections, and it will be noted from Figure 1 that two anticlinal divisions occurred in the 100 sections before compression wood formation, three in the next 100 sections, and 11 in the remaining 106 sections. Of the total of 17 daughter cells only five survived. In specimen 2, in which no compression wood was present but in which a wide growth ring (No. 15) followed a narrow growth ring (No. 14), the presence of a large number of resin canals prevented examination over as large a radial distance as in specimen 1. However, from Figure 2, it will be seen that, in the four selected radial files, no anticlinal divisions occurred in the narrow ring (44 cells wide) but there were six anticlinal divisions in the first 50 sections of the following wide ring (144 cells wide), five of the daughter cells persisting.

An attempt was made to determine the influence of the anticlinal divisions on tracheid length and micellar angle by measuring the changes that occurred in the serial tangential sections after each division (for results see Figs. 1 and 2). It should be appreciated that in Figure 1, for example, the results given are for one tracheid before the anticlinal division and for two tracheids after. Because of the few cells involved and the difficulty of determining length and angle in sections the values plotted must be considered only as trends.

(*b*) *Observations on Parent Cell Walls*

Parent cell walls surrounding radial files of tracheids were observed traversing the intercellular spaces in cross sections of compression wood as shown in Plate 1, Figures 1 and 2. Similar membranes have been demonstrated in cross sections of normal wood after careful delignification (Wardrop 1952*b*). With ordinary methods of maceration the demonstration of the parent wall was extremely difficult because in mature tissue this wall is not continuous around a number of cells in a radial file and any slight agitation was sufficient to cause the cells to separate. Parent walls were not observed at the tips of the tracheids even after most careful delignification.

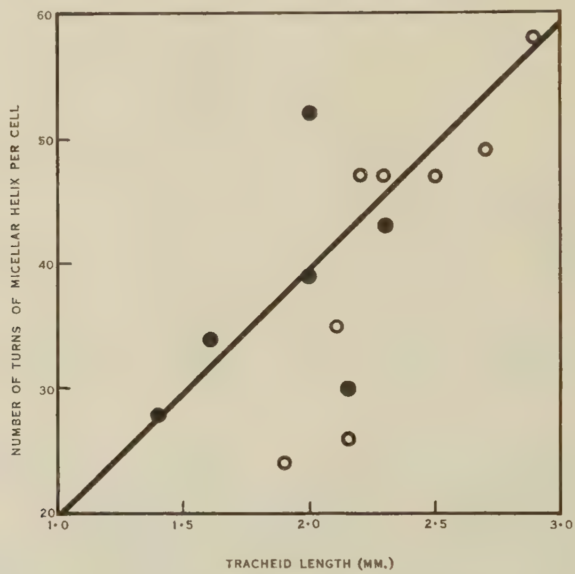

Fig. 4.—Variation in the number of turns of the micellar helix per tracheid with tracheid length in compression wood of *Cupressus macrocarpa*. ● Ring 4. ○ Ring 24.

(*c*) *Micellar Orientation in Compression Wood Tracheids*

In order to obtain further information on the helical organization of the cell walls of compression wood tracheids the number of turns of the micellar helix per cell was determined. This was done from photomicrographs of isolated cells at a magnification of 270. On the prints continuous lines were drawn parallel to the helical cracks (examples of such cracks are shown in Plate 1, Figs. 3-6) so that the total number of turns per cell could be counted. The length of each cell used for this purpose was determined from the photographs. Such determinations were carried out on macerated material from the compression wood band in specimen 1, and from compression wood taken from the fourth and 24th growth rings on the lower side of a branch of *Cupressus macrocarpa* Gordon. An increase in the number of turns with increasing tracheid length was observed in both cases, the results being shown in Figures

3 and 4. Within individual tracheids the micellar angle showed some variation, becoming greater towards the tips.

(*d*) *Development of the Secondary Wall*

For certain of the observations on cell wall formation macerated material from both normal and compression wood in specimen 1 and from slow-grown and fast-grown material in specimen 2, was examined. Many examples of bifurcated and deformed tracheid tips, suggestive of tip growth, were observed. Some of these are illustrated in Plate 1, Figures 3-6, and Plate 2, Figures 1-5. Not all of these examples were from compression wood, some were from the region of rapid growth in specimen 2. In both compression wood and normal wood tracheids from specimens 1 and 2, and in partly differentiated tracheids of *Pseudotsuga taxifolia* (Poir.) Britt., it was also observed that thickening of the cell wall was often less at the tips than in the middle (see Plate 2, Figs. 6-13) and further that where the thickening was well developed at the tips the cell lumen was connected to the primary wall by a narrow terminal canal (Plate 3, Figs. 3-8).

Secondary wall formation was also studied in branches of *Pinus radiata* D.Don, where developing compression wood tracheids in all stages of differentiation from the cambium could be observed on the lower side of the specimens (Plate 3, Fig. 9; Plate 4, Fig. 1). It can be seen from these photomicrographs that thickening of the secondary wall was incomplete for at least six to eight cells from the cambium and, further, that the rounded form of the tracheids and the intercellular spaces appeared before the development of the inner of the two layers of the secondary wall. The progress of cell wall thickening could be followed in the X-ray diffraction diagrams of serial longitudinal tangential sections 40 μ thick cut from the cambium into the xylem. The first section, containing cambium and differentiated cells in which the outer wall only was present, gave two meridional arcs on a diffuse background (Plate 4, Fig. 2). The subsequent sections which included cells with both layers of the secondary wall present gave a similar diagram but with a typical four-point diagram superimposed (Plate 4, Figure 3). These results indicate the presence of transversely oriented micelles in the outer layer and helically oriented micelles at an angle of 38° in the inner layer, thus confirming the structure of compression wood previously put forward (Wardrop and Dadswell 1950).

After X-ray examination the 40-μ sections were separately macerated and the average length of 50 tracheids determined for each consecutive section. The results obtained are set out in Table 1.

A similar procedure was carried out with specimens taken from just under the bark of normal stems of *Pseudotsuga taxifolia* collected at monthly intervals during the 1951-52 growing season. The specimens were fixed immediately after removal from the tree. Variations in cell length from the cambial zone into the xylem are again shown in Table 1. As previously observed by Wight (1933) the number of partly differentiated cells rapidly decreased as the formation of late wood began.

III. Discussion

The results obtained (see Figs. 1 and 2) show that the development of compression wood involves an increase in the number of anticlinal divisions which, in turn, is reflected in a decrease in the average tracheid length of the tissue. In addition, in a second specimen in which there was a considerable increase in radial growth rate but no compression wood, an increased number of anticlinal divisions is also involved, together with a resultant decrease in tracheid length. It is further apparent from Figure 1 that, in compression wood, the decrease in tracheid length was accompanied by an increase in micellar angle. This result, therefore, is in agreement with our earlier suggestion (Wardrop and Dadswell 1950) that the reduction in tracheid length and the increase in micellar angle associated with the development of compression wood reflect the occurrence of an increased number of anticlinal divisions in the cambium occasioned by the increased radial growth rate. They are not necessarily associated with the development of the characteristic morphological features of that tissue.

Table 1

VARIATION OF AVERAGE TRACHEID LENGTH IN SUCCESSIVE ZONES (40 μ THICK) FROM THE CAMBIUM INTO THE XYLEM

Specimen	Average Tracheid Length* (mm.)				
	Sect. 1 (Cambium)	2	3	4	5
Pinus radiata D.Don., *A*	1·40	1·47	1·63	1·74	1·87
Pinus radiata D.Don., *B*	1·41	1·45	1·51	1·55	—
Pseudotsuga taxifolia (Poir.) Britt. from Macedon, Vic., taken Nov. 1951	2·83	2·94	2·55	2·91	—
Pseudotsuga taxifolia from Macedon, Vic., taken Dec. 1951	2·43	2·53	2·63	2·59	—

* Averages of 50 measurements.

The rapid sequence of anticlinal divisions and the subsequent elimination of many of the daughter cells of these divisions, illustrated in Figures 1 and 2, effect a considerable change in the pattern made by the cambial initials on the tangential face of the developing stem. The results of the present investigation add confirmation to those given in a series of papers by Bannan and Whalley (Bannan and Whalley 1950; Whalley 1950; Bannan 1950, 1951), who have concluded that readjustment of the daughter cells following anticlinal divisions involves tip growth of the cells rather than the relatively slow symplastic readjustment proposed by Priestley (1930) and subsequently supported by Meeuse (1941).

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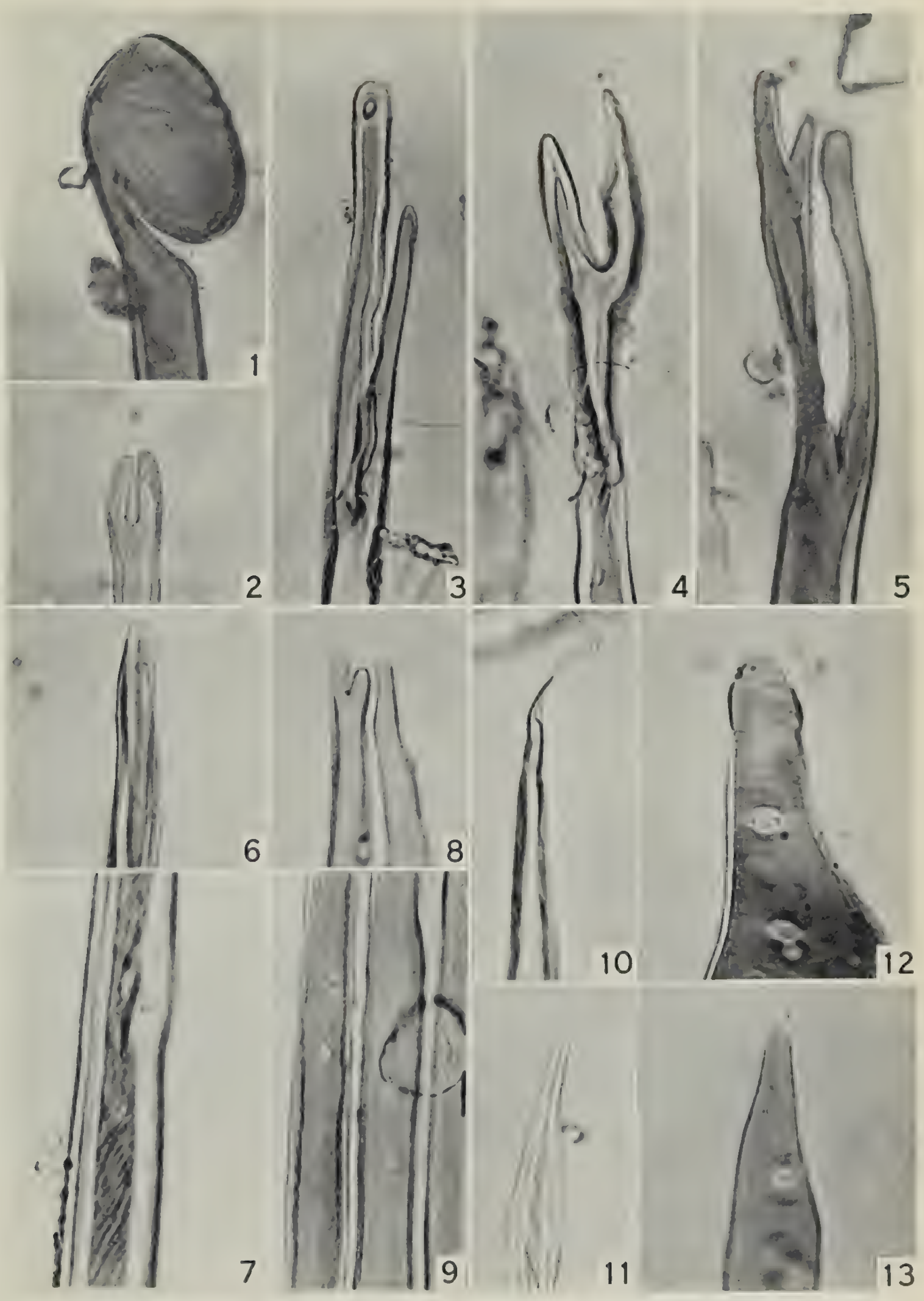

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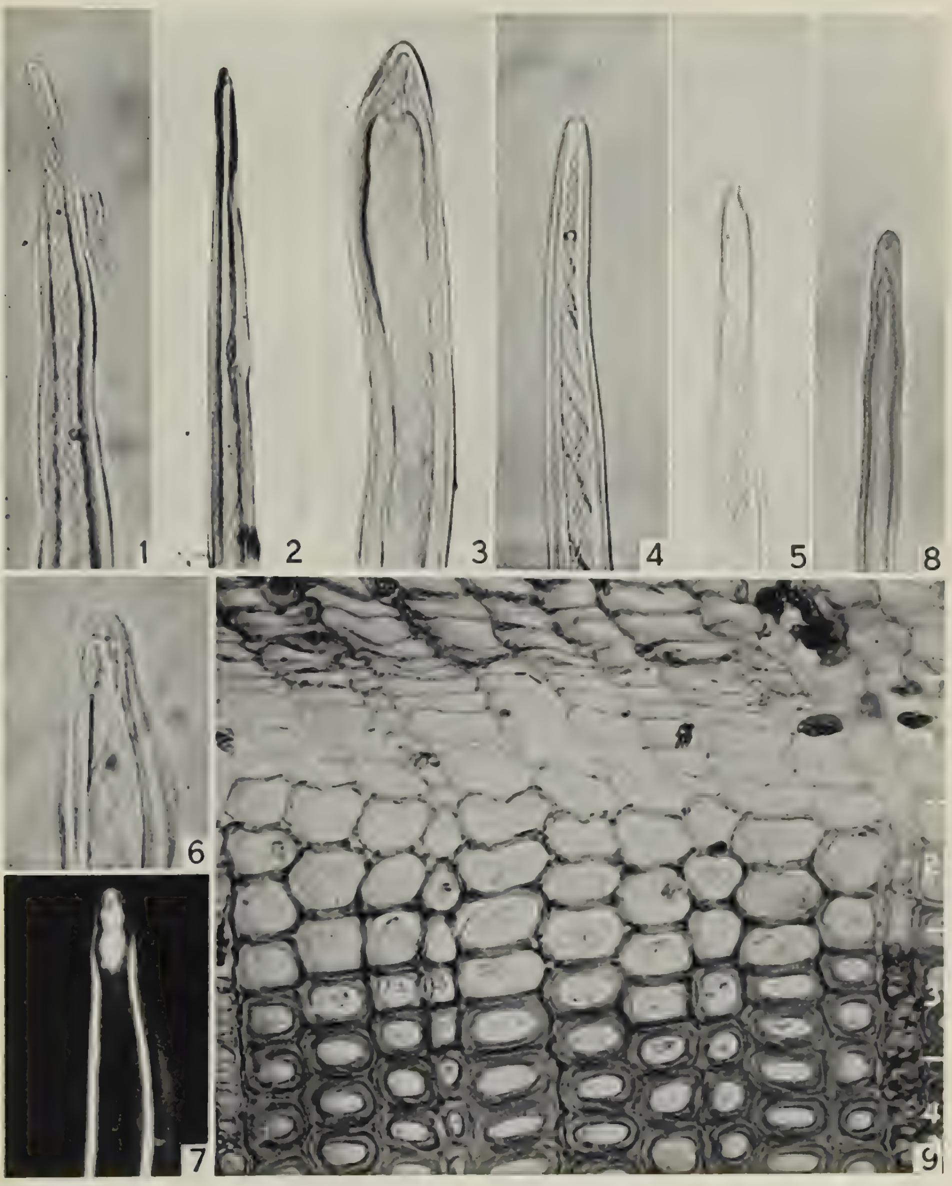

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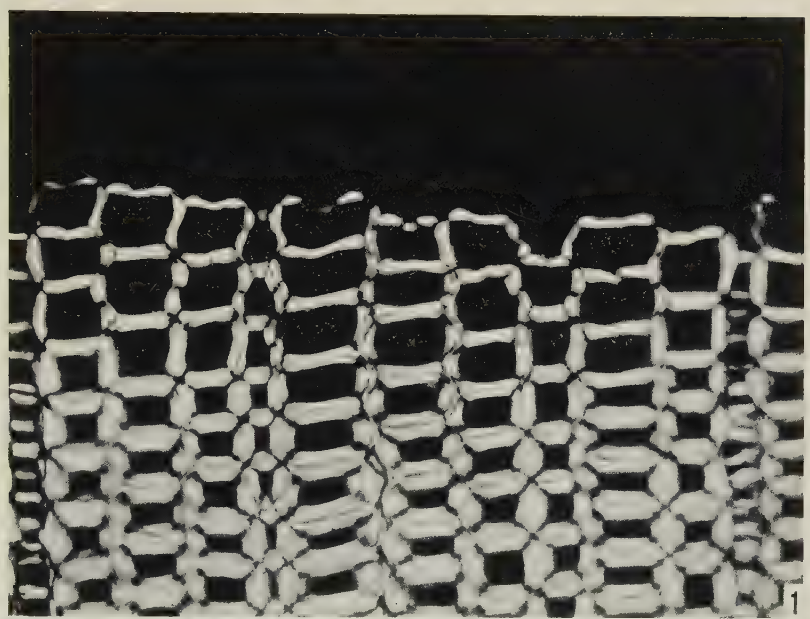

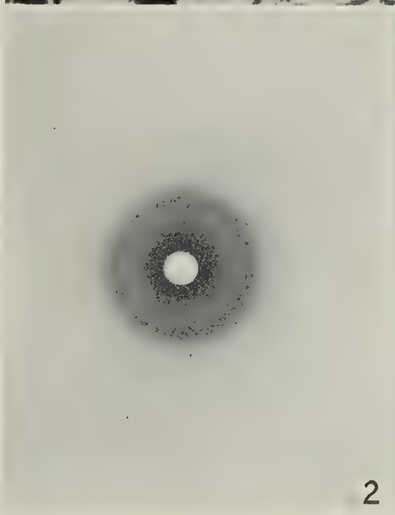

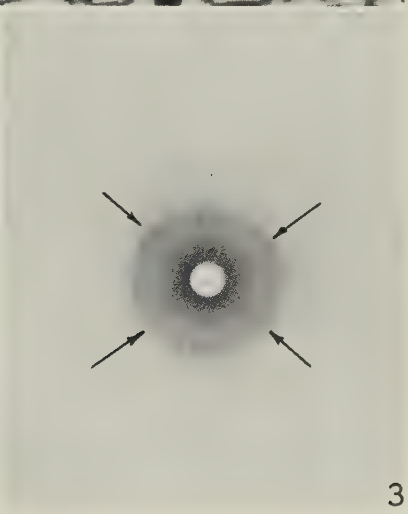

In cells in which sliding or intrusive growth* is known to occur the tips frequently show evidence of having grown against obstructions, being moulded into abnormal forms or even bifurcated (Schoch-Bodmer and Huber 1946, 1949, 1951). Impressive examples of this were found in compression wood tracheids (Plate 1, Figs. 3-6; Plate 2, Fig. 1). The frequency of occurrence of these features depended largely on the severity of the compression wood, but the fact that they were also observed in tracheids from the wide growth rings of specimen 2 in which no compression wood was present indicates that they are not a feature associated solely with the development of this tissue. Numerous examples of such bifurcated and deformed tips have since been observed in macerated material from various conifer stems. These observations thus contradict the statement of Meeuse (1941) made in support of the concept of symplastic readjustment, that in wood such as that of conifers, in which but little extra-cambial elongation takes place, no bifurcated tips are observed.

A second line of evidence supporting the concept of tip growth in conifer tracheids arises from the initial observation of Giltay (1882), subsequently supported by Priestley and Scott (1939), and by Elliot (1951), that at cell division each daughter protoplast secretes a new cell wall about itself so that the parent wall or fragments of it remain enveloping the daughter cells. The occurrence of fragments of the parent walls in the intercellular spaces of compression wood has been referred to above (Plate 1, Figs. 1 and 2) and Wardrop (1952*b*) has also demonstrated their presence in normal wood from conifers and in the ray parenchyma of angiosperms. In no instance have parent walls been observed near the tips of tracheids, suggesting that in these regions extension of the cells had ruptured them. This evidence is admittedly of a negative character, but it is consistent with the other evidence presented.

Furthermore, if tip growth occurs it is reasonable to expect that some evidence of this should be found in the anatomy of tracheid tips. Two sets of observations provide such evidence. Firstly, in partly differentiated tracheids of normal wood of *Pseudotsuga taxifolia* cell wall thickening takes place last at the cell tips so that the cytoplasm remains in contact with the extending primary wall in these regions for the greatest time (see Plate 2, Figs. 12 and 13). Secondly, in mature tracheids from both compression wood and normal wood of *Pinus pinaster* the lumen was observed to remain in contact with the primary wall at the extreme cell tip by means of a minute terminal canal (see Plate 3, Figs. 1-8), suggesting that growth, at least in the last stages of extension, was confined to the cell tips. These observations agree with those of Aldaba (1923) on the structure of the cell tips in *Linum* and *Boehmeria*, in which a similar terminal canal was observed and in which sliding growth is known to occur.

The close association of the growing cell wall and the cytoplasm is suggested in the literature by such evidence as the difficulty of plasmolysis of meristematic cells and by the fact that, although in the growing terminal cells of

* Sliding growth involves the slipping of the wall of one cell over that of a contiguous cell so that entirely new areas of contact with adjacent cells are produced. Intrusive growth involves the localized expansion of the cell wall with the newly formed parts penetrating between adjacent cells (see Eames and McDaniels 1947).

algae and fungi the cytoplasm retracts from the cell wall in regions of the filament removed from the apex, in the actual regions of growth it adheres firmly to the wall.

Thus, the rapidly changing pattern of the cambial initials, as seen in tangential section, the occurrence of bifurcated and otherwise distorted cell tips, the absence of the parent wall at the tips of tracheids, and the existence of structural features suggesting a close association between the cytoplasm and the primary wall at the tracheid tips are all points of evidence that support the concept that cell extension during differentiation proceeds by intrusive or tip growth of the cells.

Dimensional changes in the differentiation of tracheids from the cambium are closely followed by, or proceed in part simultaneously with, secondary wall formation, the completion of which constitutes the final phase of differentiation. Although it has long been recognized (Sachs 1882) that secondary thickening begins in conifer tracheids in the region of the pits, there appears to be no evidence whether thickening begins before the extra-cambial dimensional changes of differentiation are complete or whether thickening proceeds simultaneously over the whole cell length or takes place progressively. In the specimen of *Pinus radiata* with developing compression wood, relatively few cells could be seen that had undergone radial extension without formation of the outer layer of the secondary wall (cf. Plate 3, Fig. 9 and Plate 4, Fig. 1). It can be seen from Table 1 that in this and other specimens the tracheid length tended to increase in successive sections from the cambium. This clearly indicates that cell wall thickening has commenced, in these at least, before the dimensional changes of differentiation are complete. In partly differentiated tissue of *Pseudotsuga taxifolia*, tracheids were observed in which secondary thickening had obviously commenced over the major portion of the walls but not at the tips (Plate 2, Figs. 12 and 13). This also supports the view that thickening begins before the dimensional changes of differentiation are complete. In mature tracheids thickening often appeared to be less at the tips (Plate 2, Figs. 6-9) and in some specimens was quite obviously so (Plate 2, Figs. 10 and 11). Furthermore, the radial checks in the inner walls of compression wood tracheids were much less pronounced towards the cell tips, probably because of a decrease in wall thickness (see Plate 3, Figs. 1 and 2).

These radial checks, which in surface view give the appearance of pronounced striations, are parallel to the direction of micellar orientation in the inner layer and thus provide a reliable index of such orientation. By means of the method outlined above it has been shown that, in the specimens examined, the number of turns of the micellar helix per cell increases with cell length (see Figs. 3 and 4). This result at first sight appears to conflict with the relation between length and angle, used by Preston (1934) and applied by the writers to compression wood tracheids, implying a constant number of turns of the helix per cell. However, examination of individual tracheids has shown that the micellar angle varies over the cell length, usually increasing towards the tips. Phillips (1941) also has recorded variation near the flattened or irregular tracheid ends. Thus, although on the average the micellar angle

is less in longer cells, leading to the conclusion that the number of turns is constant, such a conclusion does not, in fact, hold for individual cells.

The increase in the number of turns in cells of increasing length suggests that there is a directed synthesis of cellulose in the cell wall, i.e. the synthesis is in the same direction as the orientation of the cellulose already formed. This conclusion was reached previously (Wardrop and Dadswell 1951*a*) from a study of the helical thickenings of *Pseudotsuga taxifolia* and *Taxus baccata* L., in which an increase in the number of turns of the thickenings with increasing cell length was observed. In the same way as the cell wall thickening often does not extend to the cell tips, so also the helical thickenings do not always reach the ends of the cells (Brown, Panshin, and Forsaith 1949). This evidence suggesting the progressive development of the cell wall is of interest in relation to the discussion by Thompson (1942) on the possible origin of helical structure from rectilinear growth on the surface of a cylindrical object. Furthermore, if rectilinear growth proceeds on the surface of a cone, towards its apex, the angle of inclination of the turns of the helix will tend to increase with respect to the cone axis (E. J. Williams, personal communication 1952). The similarity of this behaviour to that actually observed near the tips of tracheids is obvious. Progressive cell wall thickening is known to arise in other plant cells such as sporangiophores of *Phycomyces* and the seed hairs of cotton (Balls 1915), and of *Epilobium* (Clegg quoted by Denham 1923). In terms of submicroscopic cell wall structure the suggestion implies that the microfibrils constituting the cell wall grow in the direction of their length. This is important in relation to the possible mechanism of development of the structural units (Wardrop and Dadswell 1951*b*) and is consistent with the tendency of microfibrils to interweave in *Valonia* (Preston and Kuyper 1951) and with the observation in *Phycomyces* of microfibrils apparently attached by only one end to the cell wall (Roelofsen 1951). It is possible that helically directed synthesis of cellulose as indicated by the above observations may be involved in the so-called spiral growth of some plant cells.

In relation specifically to the formation of compression wood, the following conclusions may be drawn:

(i) The reduction in tracheid length that accompanies compression wood formation results from an increase in the number of anticlinal divisions in the cambium;

(ii) The daughter cells of these divisions do not all persist;

(iii) The subsequent extra-cambial extension of the daughter cells results from the intrusive growth between adjacent cells and the zone of growth in each cell appears to be confined to the extreme cell tips;

(iv) Secondary wall formation apparently commences before the completion of any extra-cambial readjustment, and secondary thickening proceeds progressively towards the cell tips; and

(v) At all times the cytoplasm of the lumen is in contact with the growing cell tips.

Although the observations account for the reduction in tracheid length that accompanies compression wood formation, the fact that a similar reduction can occur with changes of growth rate without the development of the particular morphological features characteristic of compression wood suggests that these features are a result of some additional influence in the stems associated with functional change. Thus, in view of the recent paper by Sinnott (1952), the development of compression wood cannot be regarded as being a purely gravitational response, but should be regarded as functional in character. Indeed, the location of compression wood in conifer stems and of tension wood in angiosperms offers an excellent example of new structural norms developed in respect to stimulus, in terms of the general principle proposed by Russel (1945) in relation to the behaviour of animals, "If . . . . normal structural and functional relations, either external or internal, are disturbed, activities will usually be set in train that are directive toward restoring structural and functional norms or establishing new norms which are adapted to the altered circumstances."

The recognition of the principle governing the development of compression wood in conifer stems does not, however, offer any explanation of those factors involved in the development of the characteristic morphology of its tracheids, which must await further, perhaps physiological, investigations.

The mechanism of cell division, the subsequent spatial readjustment of the daughter cells, and the nature of cell wall formation are all processes of general botanical interest and it is thus advantageous to consider to what extent the results obtained for compression wood are generally applicable. As there exist, in all stems in which compression wood is found, gradations from compression wood to normal wood, and as there was no evidence in the present investigation, covering both types of wood, of any differences in the mechanism of cell division, in the readjustment of the daughter cells, or in the progressive development of the cell wall, it would seem justified to assume that, in these respects, the difference between compression wood and normal wood is one of degree only. It may therefore be concluded that these processes are similar for both normal and compression wood tracheids in spite of the obvious morphological differences. Arber (1950) has discussed the considerations involved in extending to normal tissue the conclusions reached from the study of apparently abnormal tissue.

Further investigations into the causes of the morphological differences between normal wood and compression wood are planned.

IV. Acknowledgments

The authors wish to acknowledge the assistance of Miss Isabelle Cairns and Miss Valerie Biggs in compiling the data presented in Table 1 and the figures.

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Explanation of Plates 1-4

Plate 1

Fig. 1.—*Pinus pinaster.* Transverse section of compression wood, showing parts of a parent wall traversing an intercellular space. x430.

Fig. 2.—*Pinus pinaster.* Transverse section of compression wood photographed between crossed nicols, showing part of a birefringent parent wall traversing an intercellular space. x980.

Figs. 3-5.—*Pinus pinaster.* Isolated tracheids from compression wood, showing bifurcated tips. x430.

Fig. 6.—*Pinus pinaster.* A group of tracheids isolated by maceration, showing distortion of a tracheid tip in contact with a ray cell. x430.

Plate 2

Fig. 1.—*Pinus pinaster.* The distorted tip of an isolated tracheid from normal wood. x430.

Figs. 2-5.—*Pinus pinaster.* Bifurcated tips in normal wood tracheids. In Figure 5 a triple bifurcation is present. x430.

Figs. 6 and 7.—*Pinus pinaster.* The tip and middle part of a mature compression wood tracheid respectively, showing less thickening and absence of striations near the tip (Fig. 6). x430.

Figs. 8 and 9.—*Pinus pinaster.* As for Figures 6 and 7. x430.

Figs. 10 and 11.—*Pinus pinaster.* Examples of very slight secondary thickening near the tips of isolated tracheids. x430.

Figs. 12 and 13.—*Pseudotsuga taxifolia.* Semi-differentiated tracheids of normal wood, showing only the primary wall present at the cell tips. Figure 12 x980; Figure 13 x430.

Plate 3

Figs. 1 and 2.—*Pinus pinaster.* Compression wood tracheids, showing the decrease in intensity of striations towards the cell tips. x430.

Figs. 3-8.—*Pinus pinaster.* Compression wood tracheids, showing the terminal canal. Figure 7 photographed between crossed nicols. Figures 4, 5, and 8. x430. Figures 3, 6, and 7. x980.

Fig. 9.—*Pinus radiata.* A transverse section through the cambium and region of xylem differentiation. x430.

Plate 4

Fig. 1.—*Pinus radiata.* As Plate 3, Figure 9, photographed between crossed nicols. x430.

Fig. 2.—*Pinus radiata.* X-ray diffraction photograph of a 40-μ section including the cambium (zone 1, Plate 3, Fig. 9). Ni$K\alpha$ radiation. Specimen-film distance = 2.1 cm.

Fig. 3.—*Pinus radiata.* X-ray diffraction photograph of zone 4, Plate 3, Figure 9. Ni$K\alpha$ radiation. Specimen-film distance = 2.1 cm.

FURTHER HOST RANGE AND TRANSMISSION STUDIES WITH A VIRUS DISEASE OF CARROT ENDEMIC IN AUSTRALIA

By L. L. STUBBS*

[*Manuscript received June* 18, 1952]

Summary

Further host range studies with the carrot virus are described. The aphid *Cavariella aegopodii* Scopoli transmitted the virus to several non-umbelliferous species, but failed to recover the virus from these species. It was observed, however, that they were extremely unpalatable and possibly toxic to the vector.

Heteroplastic grafts between infected petunia and healthy carrot resulted in two infections in the latter species, although graft unions were not recorded.

The virus was transmitted to tobacco, but not to carrot, by sap inoculation from infected petunia.

Symptoms on all hosts except *Datura stramonium* L. were masked by rising temperatures and intensified by low-temperature conditions. Petunia, tobacco, and *D. stramonium* are regarded as good differential hosts.

From these studies it is concluded that the carrot virus is distinct from any previously described. The common name carrot motley dwarf is, therefore, proposed for the virus and the disease.

I. INTRODUCTION

In earlier studies with a virus disease of carrot in Victoria the author reported failure to transmit the virus mechanically or to infect plants outside the family Umbelliferae (Stubbs 1948). The present paper records the results of additional host range studies, which were undertaken primarily with the object of establishing the identity of the virus.

II. MATERIALS AND METHODS

The aphid *Cavariella aegopodii* Scopoli, which is the only known vector of the virus, was used exclusively in all insect transmission studies with non-umbelliferous species.

Most infections were obtained when vigorous aphids, bred on recently infected carrots, were used to inoculate test species. Groups of 10-20 aphids were normally fed on test plants, the number used varying according to the size of the plant.

A starvation period of about 24 hr., preferably at a temperature of 50-55°F., induced infective aphids to feed on species unpalatable to them in an unstarved condition.

Small feeding cages just large enough to enclose the test plants were used as it was found that aphids were restive in larger cages and frequently deserted the plant after a short preliminary feed.

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In species that proved susceptible to the virus, small, vigorously growing test plants were easier to infect and developed more severe symptoms than larger plants.

The results of many experiments, conducted over a period of 3 years, showed that successful transfers were not consistently obtained if any of the above factors were neglected. It was also found that disease symptoms were more intense and less transient when the plants were inoculated during the cooler months of the year.

TABLE 1

APHID INOCULATION EXPERIMENTS WITH NON-UMBELLIFEROUS HOSTS

Experiment	Test Species	Aphids Transferred*	Results† Infective Aphids	Non-infective Aphids	Sap Inoculation
1	Tobacco var. Hickory Pryor	10	5S/5	0/5	0/5
2	Tobacco var. Hickory Pryor	10	4L+1S/5	0/5	—
3	Tobacco var. Hickory Pryor	20	5L/5	0/5	—
4	Tobacco var. Hickory Pryor	20	8S/10	0/10	0/10
5	Tobacco var. Early Virginia	10	5L/5	0/5	—
6	Petunia var. Rose of Heaven	10	3S/5	0/5	0/5
7	Petunia var. Rose of Heaven	20	5S/5	0/5	—
8	Petunia var. Rose of Heaven	20	4L+9S/16	0/16	0/16
9	Petunia var. Rose of Heaven	20	2L+14S/16	0/16	—
10	Petunia var. Rose of Heaven	20	5L+15S/20	0/20	—
11	*Datura stramonium*	10	5S/5	0/5	0/5
12	*Datura stramonium*	10	5S/5	0/5	—
13	*Capsicum annuum*	20	10S/10	0/10	0/10
14	Nemesia	20	10S/10	0/10	—
	Spinacia oleracea var. Prickly Winter	20	0/5	0/5	—
	Sugar beet var. U.S. 33	20	0/20	0/20	—
	Tomato var. Tatura Dwarf Globe	20	0/5	0/5	—
15	*Nicotiana glutinosa*	20	0/5	0/5	—
	Nicotiana sylvestris	20	0/5	0/5	—
	Lupinus angustifolius	20	0/5	0/5	—
	Vicia sativa	20	0/5	0/5	—
	Calendula	20	0/5	0/5	—

* Infective *C. aegopodii* were bred on infected Chantenay carrots, and non-infective aphids on healthy carrots.

† The numerator refers to the number of plants infected, either locally (L) or systemically (S) and the denominator to the number inoculated. Sap inoculations, when conducted, were made with extracts from the leaves of naturally infected carrots.

III. SYMPTOMS ON APHID-INOCULATED HOSTS

Of the species inoculated with infective *C. aegopodii,* symptoms were recorded on tobacco (var. Hickory Pryor, Early Virginia), petunia (var. Rose of Heaven, Fluffy Ruffles), *Datura stramonium* L., *Capsicum annuum* L., and nemesia.

Symptoms were not observed on *Nicotiana glutinosa* L., *N. sylvestris* Speg., tomato (var. Tatura Dwarf Globe), spinach (var. Prickly Winter), sugar beet (var. U.S. 33), *Lupinus angustifolius* L., *Vicia sativa* L., and *Calendula officinalis* L.

Details of transmission experiments are recorded in Table 1. The symptoms observed on the various hosts were as follows:

(*a*) *Tobacco* (*var. Hickory Pryor*).—Local necrotic feeding lesions, frequently surrounded by a chlorotic halo, appeared on older leaves 8-13 days after inoculation. Systemic symptoms were observed 20-40 days after inoculation. They consisted of vein clearing followed by vein necrosis on young leaves, followed by severe blistering, distortion, and downward cupping of the lamina around the midrib (Plate 1, Fig. 1). Systemic symptoms did not always develop, and they decreased in severity with age and were subject to temperature masking.

(*b*) *Petunia* (*var. Rose of Heaven, Fluffy Ruffles*).—Symptom manifestation appears to be influenced by the age of the plant, air temperature, and nutrition. The most severe symptoms occurred on young plants inoculated during the winter. They consisted of tan or buff-coloured necrotic ring and line patterns, which commenced on the oldest leaves and progressed acropetally as these leaves shrivelled and died (Plate 2, Fig. 1). Symptoms were usually evident 15-18 days after inoculation.

In one experiment the growth rate of infected seedling plants was accelerated by repotting and watering with a nutrient transplanting solution. These plants made a temporary recovery under low-temperature conditions. A bright yellow vein mottle appeared after 3 weeks of symptom masking, and the original necrotic symptoms a fortnight later.

Primary feeding lesions, in the form of chlorotic blotches, or slate or buff-coloured necrotic areas, were sometimes recorded on larger plants. As with the above species they appeared only on the oldest leaves. Systemic symptoms usually commenced as a light and dark green mottle (Plate 3, Fig. 1), but necrotic shock symptoms were observed occasionally. With rising temperatures these were superseded by yellow vein symptoms, which were themselves masked by consistently high temperatures (Plate 2, Fig. 2). Severe necrotic symptoms reappeared when plants with masked symptoms were grown under low-temperature conditions (Plate 3, Fig. 2).

(*c*) Datura stramonium.—Systemic vein-clearing symptoms, restricted initially to segments on the young leaves, were observed 14-16 days after inoculation. Necrotic or chlorotic feeding lesions were sometimes recorded. Vein-clearing symptoms were followed by a mosaic mottle on the young leaves, which later became distorted and cupped downwards about the midribs. Characteristic stem distortions, consisting of constrictions alternating with necrotic eruptive areas, developed 4-6 weeks after inoculation (Plate 3, Fig. 3). The necrotic areas were brown in colour, and ultimately split longitudinally to form cankers (Plate 3, Fig. 4). The plants usually died soon after these symptoms developed.

(*d*) Capsicum annuum.—Small, red, circular feeding lesions appeared on the older leaves 12-14 days after inoculation. Systemic symptoms consisting of a light and dark green mottle, with slight blistering and distortion of the young leaves, appeared about 7 days later. Chlorotic rings appeared transiently on the older leaves and complete symptom masking occurred with rising temperatures.

(*e*) *Nemesia.*—Yellow or red vein banding and leaf distortion symptoms were recorded. Temperature masking occurred.

IV. Virus Transmission by Sap Inoculation

Following extension of the host range of the virus to non-umbelliferous species, a further attempt was made to effect its mechanical transmission. The possibility of a virus inhibitor in the sap of umbelliferous hosts was also considered as an explanation of previously reported transmission failures (Stubbs 1948).

Successful transmissions were obtained initially, when undiluted sap from experimentally infected petunia was rubbed on the leaves of young tobacco plants, previously dusted with aloxite abrasive (600 mesh). The virus is, however, difficult to transmit, as at least 50 per cent. of inoculations over a period of 2 years produced negative results. These transmission failures are attributed to subminimal concentrations of virus in the sap of the host plants.

In one experiment comparative inoculations were made with sap from petunia, tobacco, and *D. stramonium*, which were infected at the same time and grown under similar conditions. The two latter species and carrot (var. Chantenay) were used as test plants. Infections were recorded only on tobacco rubbed with petunia extract. In a later experiment sap from recently infected tobacco produced symptoms on both tobacco and *D. stramonium*. However, further comparative inoculations indicated that virus activity was greater in petunia than in tobacco extracts, and tobacco proved most suitable for use as a test species because of local lesion production.

In petunia, the highest virus concentration, as indicated by the number of local lesions on rubbed tobacco leaves, occurred in undiluted sap from young, severely affected plants. When the sap was diluted with an equal volume of water the number of local lesions and the severity of systemic symptoms were considerably reduced. Infective petunia sap diluted with an equal volume of phosphate buffer (pH 7), produced a higher percentage of systemically infected tobacco plants than sap diluted with buffer + 0.1 per cent. sodium sulphite or water.

The infectivity of petunia sap was reduced, but not destroyed, when diluted with an equal volume of sap from healthy or infected carrot leaves. It was observed, however, that undiluted carrot sap caused considerable injury on tobacco, even when the leaves were washed with water immediately after inoculation. It is possible, therefore, that the apparent reduction in infectivity of the petunia sap may be due to this factor. It was also observed that carrot sap did not cause excessive injury when inoculated on carrot leaves, yet infection did not occur.

Symptoms on mechanically inoculated tobacco leaves consisted of local lesions in the form of faint chlorotic rings, which appeared 8-10 days after inoculation. Even the most active preparations produced relatively few lesions, and then only on the oldest leaves (Plate 1, Figs. 2 and 3).

The occurrence or severity, or both, of systemic symptoms appeared to be related to the virus activity of the inoculum as indicated by the number of local lesions produced. When recorded, they were similar to, but milder than, those resulting from aphid inoculations. The removal of older leaves from tobacco plants showing mild symptoms increased symptom expression on the younger leaves, whilst topping intensified ring and line patterns on the older leaves.

Mechanically infected *D. stramonium* also produced mild leaf symptoms. The plants were less dwarfed than those infected by aphids, but necrotic stem symptoms were of similar intensity. The results of mechanical transmission experiments in which infections occurred are recorded in Table 2.

V. Attempted Recovery of Virus from Solanaceous Hosts

(*a*) *Mechanical Inoculation*

As reported above, all attempts to infect carrot by sap inoculation from experimentally infected petunia, tobacco, and *D. stramonium* have been unsuccessful.

(*b*) *Aphid Inoculation*

In attempts to recover the virus from the above solanaceous hosts, groups of non-infective *C. aegopodii* were caged on these plants for periods of 1-3 days. Even with a prior period of starvation the aphids fed only intermittently on these obviously unpalatable species. Mortality was greatest on tobacco, possibly because of the hairiness of its leaves, and least on *D. stramonium.* At the conclusion of an infection feed the surviving aphids were invariably in a weakened condition, but usually recovered after transfer to carrot or slender celery (*Apium ammi* (Jacq.) Urb.) test plants. A minimum of 10 aphids was used in each transfer and the duration of the test feed was never less than 5 days. No infections were recorded in transfers conducted over a 2-year period.

(*c*) *Dodder Linkage*

From preliminary work this method would appear to offer the greatest hope for recovery of the virus. Its use is complicated, however, by two unfavourable factors. Firstly, the species of dodder used (*C. polygonorum* Engelm., *C. campestris* Yuncker, *C. tasmanica* Engelm.) grow most vigorously during the summer when symptom masking occurs in the suspected solanaceous hosts of the carrot virus. Secondly, the dodder itself appears to become infected. In one experiment *C. polygonorum* parasitizing infected petunia developed stem distortion and brown necrotic streak symptoms similar to those observed on *D. stramonium.* After the development of symptoms haustorial production ceased, and the plants seeded prematurely, thus preventing linkage with test plants. On another occasion, however, linkage was effected between infected

TABLE 2

MECHANICAL INOCULATION EXPERIMENTS WITH CARROT VIRUS

Exp. No.	Test Species	Inoculation Details*	Results†
1	Tobacco var. Hickory Pryor	(*a*) Undiluted sap from aphid-inoculated petunia	2L+2S/5
		(*b*) Undiluted sap from aphid-inoculated tobacco	0/5
		(*c*) Undiluted sap from aphid-inoculated *D. stramonium*	0/5
	D. stramonium	(*a*) As above	0/5
		(*b*) As above	0/5
		(*c*) As above	0/5
	Carrot var. Chantenay	(*a*) As above	0/5
		(*b*) As above	0/5
		(*c*) As above	0/5
	Tomato var. Tatura Dwarf Globe	(*a*) As above	0/5
		(*b*) As above	0/5
		(*c*) As above	0/5
2	Tobacco var. Hickory Pryor	(*a*) Undiluted sap from aphid-inoculated tobacco	0/5
		(*b*) As for (*a*) but diluted 1/1 with 0·1% Na_2SO_3	0/5
	D. stramonium	(*a*) As above	2S/5
		(*b*) As above	0/5
	Petunia var. Rose of Heaven	(*a*) As above	0/5
		(*b*) As above	0/5
3	Tobacco var. Hickory Pryor	(*a*) Undiluted sap from recently infected petunia	8S/10
		(*b*) Undiluted sap from long-infected petunia with severe symptoms induced by low temperatures after a period of masking	2L/10
		(*c*) As for (*a*) but diluted 1/1 with sap from carrot leaves	3S/10
		(*d*) As for (*a*) + diatomaceous earth	0/10
		(*e*) Undiluted sap from infected carrot leaves	0/20
4	Tobacco var. Hickory Pryor	(*a*) Undiluted sap from recently infected petunia diluted 1/1 with phosphate buffer (pH 7)	10S/10
		(*b*) As for (*a*) diluted 1/1 phosphate buffer +0·1% Na_2SO_3	2L+8S/10
		(*c*) As for (*a*) diluted 1/1 with water	2L+8S/10

* All test plants were dusted with aloxite abrasive before inoculation.

† The numerator refers to the number of plants infected, either locally (L), or systemically (S), and the denominator to the number inoculated.

and healthy petunia plants before symptoms developed on the dodder. In this experiment the test plants became infected. All of the above species of dodder grow profusely on both petunia and carrot, but fail to grow on *D. stramonium.*

This method of transmission is being further investigated.

(*d*) *Graft Transmission*

Following the report by Nickell (1948) of successful grafts between unrelated plant species, attempts were made to graft infected petunia to carrot.

In the first experiment holes were drilled to a depth of approximately ½ in. in the root crowns of young, healthy carrots with a flame-sterilized metal drill ⅛ in. in diameter. The outer bark tissues were removed from the basal portions of healthy and infected petunia tip-cuttings, which were slightly more than ⅛ in. in diameter. These scions were then inserted into the holes in the carrot roots and surface-sealed with melted microcrystalline paraffin wax. The grafted plants were held in a warm humid chamber for 3 weeks and then removed to a cool greenhouse for observation.

One carrot plant of six grafted developed typical virus symptoms. Infection was subsequently confirmed by aphid transmission to slender celery.

In a subsequent experiment the cleft grafting technique was used. Petunia scions were inserted into the carrot crown tissues immediately below the junction of the petioles with the roots. Each petunia scion was pierced by a needle with a thread of candlewick cotton attached. The loose end of the cotton was immersed in a reservoir of nutrient solution throughout the experiment. As in the previous experiment, graft unions were not observed, but one infection was recorded. The control plants remained healthy.

VI. Persistence of the Virus in C. aegopodii Fed Alternately on D. stramonium and Carrot

It has been reported elsewhere (Stubbs 1948) that the carrot virus is extremely persistent in its vector. It would be expected therefore that indirect evidence regarding the identity of the virus transmitted to solanaceous hosts would accrue from a study of its persistence in the vector.

With this objective aphids bred on infected carrot were transferred serially to carrot and *D. stramonium* test plants. Initially, groups of 20 infective aphids were starved for 24 hr. before the first test feed. After a test feed of 4 hr. they were transferred to moist filter pads in cellophane-covered petri dishes and starved for the 20 hr. preceding the next transfer. In the second serial transfer those aphids which had fed on carrot test plants were transferred to *Datura* and vice versa. This alternation of short test feeds on carrot and *Datura,* which continued for the duration of the experiment, was designed to minimize the apparent toxic effect previously observed on aphids fed on solanaceous species for long periods. The experiment was continued until most of the aphids had died.

It was observed that aphids lost vitality in one test feed on *Datura* but regained it in the following feed on carrot, that infections occurred on both

carrot and *Datura* in each serial transfer throughout the experiment, that in all except transfers 1 and 2 a higher percentage of infections occurred in *Datura* than in carrot, and that the symptoms observed on *Datura* even after the aphids had been removed from the infection source for 8 days were identical with those described above. The experimental details are recorded in Table 3.

TABLE 3

ALTERNATE TRANSFERS TO CARROT AND *DATURA* WITH INFECTIVE *C. AEGOPODII*

Transfer No.	Days Since Aphids Fed on Virus Source	Infections Recorded*	
		Carrot	*Datura*
1	1	4/4	3/4
2	2	3/4	3/4
3	3	2/4	4/4
4	4†	2/4	4/4
5	7	3/4	4/4
6	8	1/4	4/4

* The numerator refers to the number of plants infected and the denominator to the number inoculated.

† Aphids removed from plants at conclusion of test feed and starved over week-end.

VII. DISCUSSION

The consistent transmission by *C. aegopodii* of a virus from infected carrot to several non-umbelliferous species does not in itself provide sufficient evidence to identify this virus with the carrot virus. The fact that the vector failed to recover the virus from solanaceous species could be interpreted as evidence that it is distinct from the carrot virus, or that the latter underwent some chemical or structural change in these hosts. However, from further evidence presented it seems unlikely that such deductions would be plausible.

Failure of the vector to acquire the virus from unpalatable species could be due to its inability to obtain a minimal infecting dose in a series of short, starvation-induced feeds. It is also possible that substances toxic to the vector occur in these hosts, which may reduce temporarily its ability to transmit. In the experiment described above, where infective aphids were fed alternately on carrot and *Datura* test plants, it was observed that aphids fed on *Datura* for 4-hour periods were in a weakened condition. This was reflected in the lower percentage of infections on carrot in the succeeding transfer. A similar example of unpalatability or toxicity, or both, to the vector was reported earlier for an umbelliferous species, *Conium maculatum* L. (Stubbs 1948). The virus was recovered with difficulty from this species.

The only direct evidence of virus recovery was obtained by means of heteroplastic grafts between experimentally infected petunia and healthy carrot. The single infections, recorded in each of two experiments conducted under insect-

free conditions and with equal numbers of controls, may have resulted from temporary unions of the stock and scion tissues. This evidence must, however, be regarded as inconclusive.

The contention that only one virus, the carrot virus, is responsible for experimental infections produced in non-umbelliferous hosts, is supported by the following indirect evidence:

(1) The same range of symptoms has been produced on each host by infective aphids collected in many different localities.

(2) Symptom masking occurs on these hosts, as on umbelliferous hosts, when exposed to high-temperature conditions.

(3) Infections were produced on *Datura* and carrot by aphids removed from an infection source for as long as 8 days. It would appear improbable, therefore, that any other virus could be involved, and reasonable to accept the above experimentally infected species as alternate hosts of the carrot virus. Tobacco, petunia, and *D. stramonium* may be regarded as good differential hosts, in the event of the virus being recorded outside Australia.

The virus has characteristics in common with viruses of the sugar beet yellows (Watson 1940) or sugar beet yellow-net (Sylvester 1948) type. These viruses are characterized by their restricted host range and long persistence in their vectors. They are difficult to transmit, or are non-transmissible by mechanical methods of inoculation, and appear distinct from non-persistent aphid-transmitted viruses. In regard to nomenclature, therefore, it is considered undesirable to use the name carrot mosaic for the carrot virus. As the name carrot yellows is already used in America for a disease caused by the aster yellows virus (Severin 1932), it is proposed to apply the name carrot motley dwarf virus to the disease. The adjective motley, meaning "diversified in colour," is used to describe autumn tinting of the foliage. Dwarfing and foliage tinting are regarded as the main diagnostic features of the disease.

VIII. Acknowledgments

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Explanation of Plates 1-3

Plate 1

Fig. 1.—Tobacco plant systemically infected with carrot virus. Local feeding lesions are present on older leaves.

Fig. 2.—Local lesions on tobacco leaf 8 days after mechanical inoculation with sap from artificially infected petunia.

Fig. 3.—The same leaf as in Figure 2 decolourized in alcohol, and stained with a solution of iodine in potassium iodide.

Plate 2

Fig. 1.—Left, healthy petunia seedling. Right, plant infected by aphid inoculation, showing severe necrotic symptoms on lower leaves.

Fig. 2.—Yellow vein "recovery" symptoms on artificially infected petunia.

Plate 3

Fig. 1.—Early mottle symptoms on large, systemically infected petunia plant.

Fig. 2.—Severe necrotic symptoms on petunia induced by low-temperature conditions, following a period of high-temperature symptom masking.

Fig. 3.—Left, healthy *D. stramonium*. Centre, infected by sap inoculation from artificially infected tobacco. Right, infected by aphid inoculation, showing stem distortion symptoms.

Fig. 4.—Portion of stem from infected *D. stramonium* plant showing "canker" symptoms.

VIRUS DISEASE OF CARROT IN AUSTRALIA

1

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VIRUS DISEASE OF CARROT IN AUSTRALIA

VIRUS DISEASE OF CARROT IN AUSTRALIA

1 2 3 4

FUNGAL CELLULASES

I. GENERAL PROPERTIES OF UNPURIFIED ENZYME PREPARATIONS FROM *ASPERGILLUS ORYZAE*

By M. A. JERMYN*

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Summary

The culture filtrate from *Aspergillus oryzae* contains enzymes capable of depolymerizing sodium carboxymethyl cellulose (SCMC) and of splitting all β-glucosides tested. These enzymes are produced by the mould in the absence of cellulose or any other carbon source containing β-glucosidic linkages.

The action of the enzyme degrading SCMC (the C_x enzyme of Reese, Siu, and Levinson 1949) and the β-glucosidase activities were followed iodometrically. The β-glucosidase action was also followed colorimetrically using *p*-nitrophenyl-β-glucoside as a substrate.

The variation in the activity of the C_x enzyme with temperature, pH, and enzyme and substrate concentrations, and in the presence of inhibitors, was measured and some kinetic data are also given for the β-glucosidase activities. On the basis of these results it is concluded that the physicochemical properties of SCMC solutions make this compound an unsatisfactory substrate for kinetic studies. The true pH optimum of the C_x enzyme is about 3.5; the apparent temperature optimum is very strongly influenced by reaction time.

The behaviour of the C_x enzyme towards metal-complexing, oxidizing, and reducing agents can be explained if it is activated by ferric ions but the method of enzyme estimation used and the complexing properties of sodium carboxymethyl cellulose make proof of this hypothesis difficult. The enzyme activity is also strongly affected by various organic bases, notably caffeine and quinine. The behaviour of a β-glucosidase (salicinase) towards various reagents affecting the C_x enzyme has also been tested.

I. INTRODUCTION

Studies that have been commenced in this laboratory on the biochemistry of the cellulolytic fungi required the development of methods for the estimation of cellulase and related enzymes. For this purpose it seemed that the pool of enzyme protein from the culture filtrate of *A. oryzae,* which was already available (Crewther and Lennox 1950; Gillespie and Woods, unpublished data), might serve as a suitable source of material. This paper embodies a description of the methods finally adopted for enzyme estimation and their application to determining the general properties of the unpurified enzyme preparation.

It has long been known that taka-diastase and other products of *A. oryzae* and related species contain an active β-glucosidase ("mould emulsin"). Although the *flavus-oryzae* group of the aspergilli are usually classified among the non-cellulolytic fungi (Marsh *et al.* 1949), strong cellulase activity was found

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in culture filtrates by Grassmann and Rubenbauer (1931) and Freudenberg and Ploetz (1939). Modern concepts of enzyme specificity having reduced much of the earlier work on "cellulase" to a purely descriptive value, the general uncertainty that has so often in the past surrounded the definition of the substrate and activity of "cellulase" makes it worth while to confirm and extend such reports.

Its was not until the observations of Freeman, Baillie, and MacInnes (1948) on sodium carboxymethyl cellulose (SCMC) that a soluble substrate was readily available which permitted attack on the β-glucosidic linkages of a cellulose-like material in a homogeneous system. SCMC has been used in the work of Holden and Tracey (1950), Tracey (1950; 1951), Reese, Siu, and Levinson (1949), and Levinson and Reese (1950). It is water-soluble at D.S.* 0.4 and upwards. Calculation shows that at D.S. 0.4 about 40 per cent. and at D.S. 0.6 about 20 per cent. of the β-glucosidic linkages of the SCMC chains are between unsubstituted glucose residues. The figures of Holden and Tracey for the limiting breakdown of SCMC by snail cellulase are in agreement with the idea that these are the linkages attacked.

The breakdown of SCMC is thus an index of the presence of an enzyme that is capable of hydrolysing polymeric β-glucosidic linkages. Reese, Siu, and Levinson (1949) point out that this enzyme is not identical with "cellulase" as it has been understood in the past, since it is produced by fungi incapable of attacking native cellulose although capable of attacking degraded and solubilized celluloses. They therefore postulate that apparent "cellulase" activity in fungi is really the combined activity of two enzymes, "C_1," produced by cellulolytic organisms and capable of bringing native cellulose, perhaps by breaking cross links, into a form sufficiently soluble to be attacked by "C_x," an enzyme produced by both cellulolytic and many non-cellulolytic organisms. Reese and Downing (1951) have shown that the aspergilli can be broadly divided into two sections—those possessing "C_x" and "C_1" and able to attack native cellulose, and those possessing "C_x" alone and able to attack only soluble cellulose derivatives. The *flavus-oryzae* group falls into the latter section.

II. Materials

(*a*) *p-Nitrophenyl-β-D-glucoside*

This is as prepared from p-nitrophenol and β-glucose penta-acetate by the method of Helferich and Schmitz-Hillebrecht (1933) as white crystals of m.p. 164°C. (Goebel and Avery (1929) give 165°C.).

(*b*) *Modified Celluloses*

Reprecipitated cellulose was prepared from cotton wool dissolved in phosphoric acid; cellodextrin by the ethanol fractionation of cotton wool degraded by solution in 72 per cent. sulphuric acid to a water-soluble form. The cellodextrin used had a degree of polymerization of about 100.

* D.S. = degree of substitution = average number of carboxymethyl groups per glucose residue.

(*c*) *Sodium Carboxymethyl Cellulose*

A sample of Cellofas B (blend 874, medium viscosity) was obtained from Imperial Chemical Industries Ltd., Steventon, Ayrshire, through the kind offices of Dr. G. G. Freeman. The degree of substitution was 0.65 as determined by the sodium salt method of McLaughlin and Herbst (1950).

Stock solutions of SCMC were freshly made up at 1 per cent. strength by one of two methods as required:

(i) The SCMC was thoroughly mixed with 1/20 of the final volume of distilled water and allowed to stand overnight. The resulting jelly was carefully diluted by stirring in successive small additions of water until a clear viscous solution resulted and this was made up to volume.

(ii) The SCMC was beaten up with a little less than the required volume of water at about 80°C. in a Waring Blendor until the mixture was homogeneous and the solution allowed to cool and made up to volume.

Solutions made up by method (ii) were attacked by the mould enzyme about twice as fast as those made up by method (i) but were unstable, the rate of attack falling off to the lower level after 2-3 days. All solutions aged slowly at room temperature with separation of gel-like material and increased resistance to enzyme attack. Storage in a refrigerator accelerated these undesirable changes.

(*d*) *"Mould Enzyme"*

The general properties of this material have been described by Crewther and Lennox (1952). A single batch of about 30 g. was used in all experiments. It contained some 10 per cent. of carbohydrate material as determined by the anthrone method of Morris (1948). Hydrolysis with 3 per cent. nitric acid and paper chromatography showed roughly equal amounts of mannose and galactose. This carbohydrate was not hydrolysed by the enzyme since prolonged incubation of enzyme solutions revealed no rise in reducing value or liberation of free sugars.

III. Methods

(*a*) *Estimation of* C_x *Enzyme*

The method used is based on the iodine-reducing action of the aldehyde groups liberated by the enzyme (see Hinton 1940; Jermyn and Tomkins 1950, for the application of similar methods to pectin). As standard conditions, SCMC solution (1 per cent.), McIlvaine citrate-phosphate buffer (pH 5.0), and enzyme solution are mixed in the volume ratio 10 : 3 : 2 and incubated at 87°C. The iodine consumption (ml. 0.1N I_2) when 10 ml. of 0.1N I_2 reacts with 15 ml. of the digest under alkaline conditions is an index of the reducing groups liberated by the enzyme after any period of time. The total hydrolysis of 1 ml. of 1 per cent. SCMC solution should liberate reducing groups nearly equivalent to 1 ml. of 0.1N I_2; multiplication of the figure for iodine consumption by 10 therefore gives an approximate estimate of the percentage of glucosidic linkages broken in the substrate.

When iodine consumption is plotted against time a smooth curve is obtained. The tangent at the origin is estimated by fitting a parabolic expression to the curve and the slope expressed as ml. of 0.1N I_2 consumed per hour. This initial rate of iodine consumption is taken as the measure of enzyme activity.

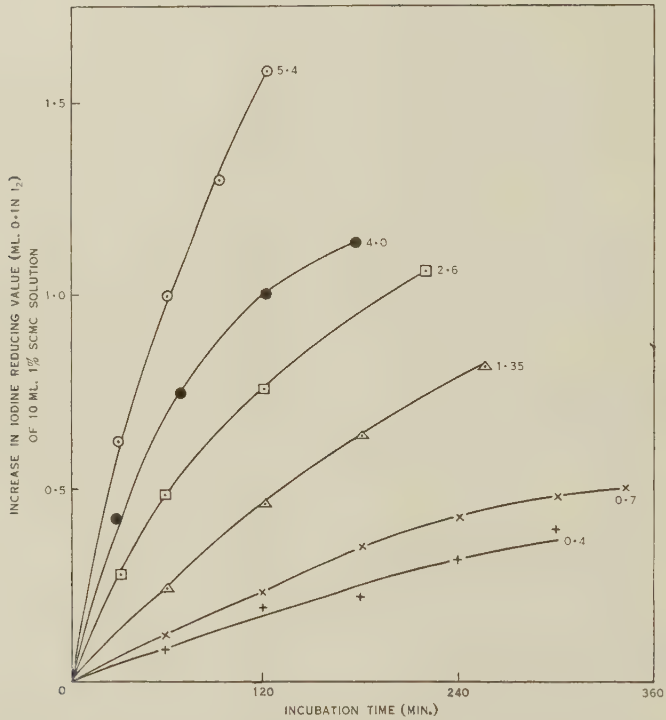

Fig. 1.—Progress curves for the hydrolysis of SCMC by crude *A. oryzae* enzyme under standard conditions. Final enzyme concentrations are indicated as mg./ml.

Figure 1 shows that the initial slope of the iodine consumption curve is very little different from that of the straight line joining the zero and 30-min. points over a wide range of enzyme concentrations. The iodine consumption during the first 30 min. of incubation was therefore routinely used ("short method") as a measure of enzyme activity. This simplified procedure is not valid under conditions departing very widely from those used as the standard.

(*b*) *Iodometric Estimation of β-glucosidase*

The iodometric method can be used to determine β-glucosidase activity using salicin, aesculin, cellobiose, β-methyl glucoside, *p*-nitrophenyl-β-glucoside, and cellodextrin as substrates. The procedure is that used in the short method for the C_x enzyme with modifications to allow for the peculiarities of individual substrates. With phenolic glucosides the volume of iodine consumed is not a measure of the glucose liberated since the liberated phenols also react with hypoiodite solutions. The method is therefore not directly quantitative but

is reproducible under the conditions stated, and the amount of iodine consumed can therefore be used as a purely comparative index of enzyme activity.

(*c*) *Colorimetric Estimation of β-glucosidase*

The optical density at pH 8.5-9.0 due to liberated *p*-nitrophenol is determined after incubation of the enzyme at 28°C. with 0.001M *p*-nitrophenyl-β-glucoside. Provided the breakdown of substrate does not exceed about 20 per cent., optical density after 20 min. incubation is proportional to enzyme concentration. The standard pH of incubation for routine activity measurements is 5.0. This procedure is essentially the same as those of all authors who have followed Aizawa (1939) in using nitrophenyl glycosides as enzyme substrates.

(*d*) *Visual Methods of Estimating SCMC Breakdown*

When three volumes of 1 per cent. SCMC solution are mixed with one volume of 10 per cent. lead acetate solution the mixture sets immediately to a stiff gel. If the SCMC has been previously partially degraded by small amounts of enzyme for varying periods the appearance passes through the stages of thick gel, thin gel broken up on shaking, curdy precipitate, and flocculent precipitate. Finally only the faint residual precipitate due to the enzyme protein is left. The breakdown has in general progressed well into the curdy precipitate stage before there is a measurable increase in iodine consumption.

Another visual index of SCMC breakdown is given by its power of forming complexes with iodine. On adding an equal volume of 0.1N iodine solution to a 1 per cent. solution of SCMC, making alkaline, and reacidifying, a thick blue-black precipitate is formed in the solution. With increasing degradation of the SCMC this passes to a blue-black solution and then through brown-black and dark brown solutions to the normal iodine colour. The change is complete at about 1.0 per cent. breakdown. This behaviour complicates the iodometric method of estimating C_x activity although the iodine-SCMC bonding is not sufficiently strong to affect the accuracy of the titration if this is done slowly with vigorous shaking in the first stages.

Both these tests are sufficiently sensitive to detect the presumably microbiological deterioration of SCMC solutions that have been standing for some time at room temperature.

IV. Enzyme Characteristics

(*a*) *Enzyme Production and Culture Conditions*

The strain of *A. oryzae* (292-4795) used in this laboratory by Maxwell (1952) for production of the crude enzyme was grown in shake culture (120 cycles/min.) in parallel with *Stachybotrys atra*, a recognized cellulolytic organism. Three carbon sources were used—sucrose, SCMC, and ground cotton wool (60 mesh), the mineral source being the Waksman-Carey medium as modified by Fahraeus (1947) with the addition of 12 μg./l. of biotin required for the optimal growth of *S. atra*. The growth of *S. atra* on all three carbon sources was good, that of *A. oryzae* good on sucrose, scanty on SCMC, and

only demonstrable by staining and microscopy on ground cotton wool. The results are incorporated in Table 1. The β-glucosidase was measured colorimetrically, and protease by the gravimetric method of Crewther (1952). The activity values for β-glucosidase are given on a subjective scale of visual intensity; those for C_x and protease may be taken as arbitrary units in this context. Values less than 1.0 for protease activity are not significant.

TABLE 1

GROWTH AND ENZYME PRODUCTION BY *ASPERGILLUS ORYZAE* AND *STACHYBOTRYS ATRA* GROWN IN SHAKE CULTURE AT 28°C. ON MODIFIED WAKSMAN-CAREY MEDIUM

Carbon Source	Period of Incubation (days)	Sucrose (2% w/v)		SCMC (2% w/v)		Cellulose (ground cotton wool) (2% w/v)	
		A. oryzae	*S. atra*	*A. oryzae*	*S. atra*	*A. oryzae*	*S. atra*
C_x enzyme	3	–	–	0·35	0·23	0·04	0·24
β-Glucosidase		±	–	+	++++	+	+++
C_x enzyme	5	–	–	0·47	0·42	0·27	0·06
β-Glucosidase		–	±	++++	+	±	+
C_x enzyme	10	–	–	0·11	0·57	0·15	–
β-Glucosidase		–	–	++++	++	+	++
Protease		0·2	2·3	5·3	1·2	1·7	1·1
C_x enzyme	16	–	0·24	0·44	0·57	–	0·30
β-Glucosidase		–	–	+++	+++	±	++
Protease		0·5	1·0	6·7	0·6	1·2	0·0
C_x enzyme	21	0·10	0·59	0·34	Not done	–	–
β-Glucosidase		+	±	++	++	±	±

(*b*) *General Properties of the Enzymes*

The complex nature of the mixture in which the C_x enzyme and β-glucosidase of *A. oryzae* appear as minor components render certain lines of investigation unprofitable unless extensive purification can be accomplished. No work therefore has been carried out on inactivation by heat and pH change or on the nature of the end-products of enzyme action. In the first case, attack by proteases renders any conclusions worthless, and in the second, enzymes are present capable of attacking any end-products other than simple monomers. Nevertheless, other data allow conclusions on these subjects to be drawn in some instances. Chromatographic examination of the products of hydrolysis of cellodextrin by crude *A. oryzae* enzyme showed that in the early stages of the hydrolysis products containing one, two, and three glucose residues were about equally prominent. This observation is incompatible with a single mechanism of hydrolysis with cellobiose units being removed from the polymeric β-glucoside chain and subsequently being hydrolysed to glucose, but is compatible with the simultaneous action of more than one mode of hydrolysis.

The conclusions of Reese, Siu, and Levinson (1949) on the existence of at least two enzymes (C_1 and C_x) in the cellulase complex are borne out by the behaviour of the *A. oryzae* enzyme. Although attack on SCMC and cellodextrin is vigorous there is little or no attack on native cellulose or insoluble derived celluloses. Table 2 illustrates these points.

TABLE 2

ATTACK ON CELLULOSE AND CELLULOSE DERIVATIVES BY *A. ORYZAE* ENZYME

Substrate (500 mg.) in 15 ml. pH 5.0 McIlvaine buffer containing 0.2 per cent. sodium benzoate; enzyme concentration 5 mg./ml.; incubation temperature 37°C.; incubation time 24 hr.

Substrate	Ground Cotton Wool (60 mesh)	Ground Whatman No. 1 Filter Paper (60 mesh)	Ground Cellophane (60 mesh)	Reprecipitated Cellulose	Cellodextrin	SCMC
Reducing groups liberated (as mg. glucose)	0·95	1·08	0·56	0·95	39·5	28

No statistically significant effect could be demonstrated on the strength of cotton sewing thread incubated in 10 per cent. enzyme solution at pH 5 at room temperature and 37°C. for periods up to a week. None the less, electrophoretic and chromatographic experiments have shown a marked tendency for break-up of the filter paper to occur at places where the enzyme has been in contact with the paper for considerable periods. Miss M. E. Maxwell has reported to the author that, during the filtering of certain batches of culture, rapid disintegration of the filter papers was observed. It is possible therefore that a little C_1 enzyme was originally present but was more liable to storage and protease action than the C_x enzyme.

Besides the compounds with β-glucosidic linkages that were further investigated the following β-glucosides were split by the enzyme: rutin, quercitrin, arbutin, phenyl-β-glucoside, α-naphthyl-β-glucoside. The phenyl and naphthyl-β-glucosides were split at about the same rate as salicin. Rutin, quercitrin, and arbutin behaved rather like aesculin but because of erratic results in the iodometric method were not further investigated.

Holden and Tracey (1950) found that the attack of snail cellulase on SCMC did not proceed to completion but only to a point corresponding to the breakage of all linkages between two adjacent unsubstituted glucose residues. Calculations following the method of Spurlin (1939) show that in a SCMC of D.S. 0.65 about 23 per cent. of the β-glucosidic linkages should be between such unsubstituted glucose residues. The degree of breakdown of cellodextrin should on this theory be 100 per cent. This analysis assumes an enzyme of the α-amylase or polygalacturonase type which attacks all the linkages of a straight-chain substrate at random. Enzymes with properties similar to β-amylase, splitting the cellulose chain to cellobiose units, such as those studied

by Levinson, Mandels, and Reese (1951) would give different results when acting alone but the presence of active cellobiase in the *A. oryzae* enzyme would give the same final results in both cases.

It was found that only by using very strong enzyme solutions over relatively short periods was a figure for the limiting decomposition of SCMC attained. Similar phenomena were observed using cellodextrin as substrate. The highest degree of breakdown observed with cellodextrin was 57 per cent., using an enzyme concentration of 24 mg./ml. Increasing difficulties due to the large blanks caused by the iodine consumption of the enzyme solutions prevented the method being pushed beyond this point but the steady increase in maximum decomposition with increasing enzyme concentration made it probable that 100 per cent. decomposition was attainable if the enzyme concentration were raised sufficiently. Figure 2 illustrates these points.

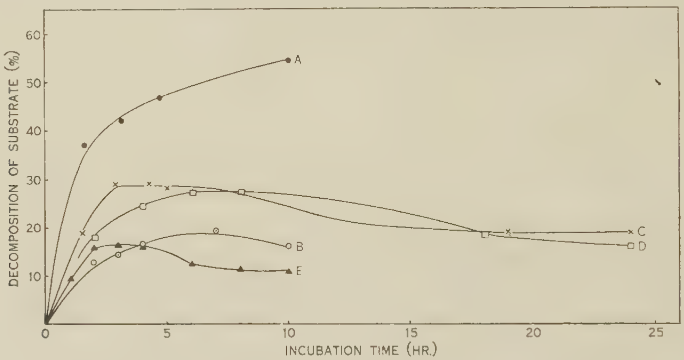

Fig. 2.—Effect of various factors on the breakdown of cellodextrin and SCMC by the *A. oryzae* enzyme, under the standard conditions with toluene and 0.2 per cent. sodium benzoate added.
A, cellodextrin 0.33 per cent., enzyme 24 mg./ml.
B, cellodextrin 0.67 per cent., enzyme 6 mg./ml.
C, SCMC 0.13 per cent., enzyme 25 mg./ml.
D, SCMC 0.13 per cent., enzyme 15 mg./ml.
E, SCMC 0.67 per cent., enzyme 6 mg./ml.

The addition of sodium benzoate and toluene inhibited gross microbiological contamination for 24 hr. at least, and destruction of substrate through this agency was negligible. The disappearance of iodine-reacting material could therefore best be explained if synthesizing enzymes were in competition with the hydrolytic enzymes and the effects of the synthesizing enzymes became apparent when the hydrolytic enzymes were inactivated. Interference with the normal course of hydrolysis would also occur in the presence of trans glucosidase systems, and Pazur and French (1951) have in fact found a trans glucosidase in *A. oryzae* culture filtrates that acted on α-glucosidic substrates. Takano and Miwa (1950) have shown that the β-glucosidase and glucotransferase activities of apricot emulsin are probably identical. Incubation at room temperature of the enzyme (2.5 mg./ml.) with 1 per cent. solutions of glucose and cellobiose followed by paper chromatography of samples withdrawn at

intervals showed that in the cellobiose solutions, besides glucose and residual cellobiose, higher molecular weight material, presumably oligosaccharides, could be demonstrated between 18 and 90 hr. of incubation. The matter was not investigated further.

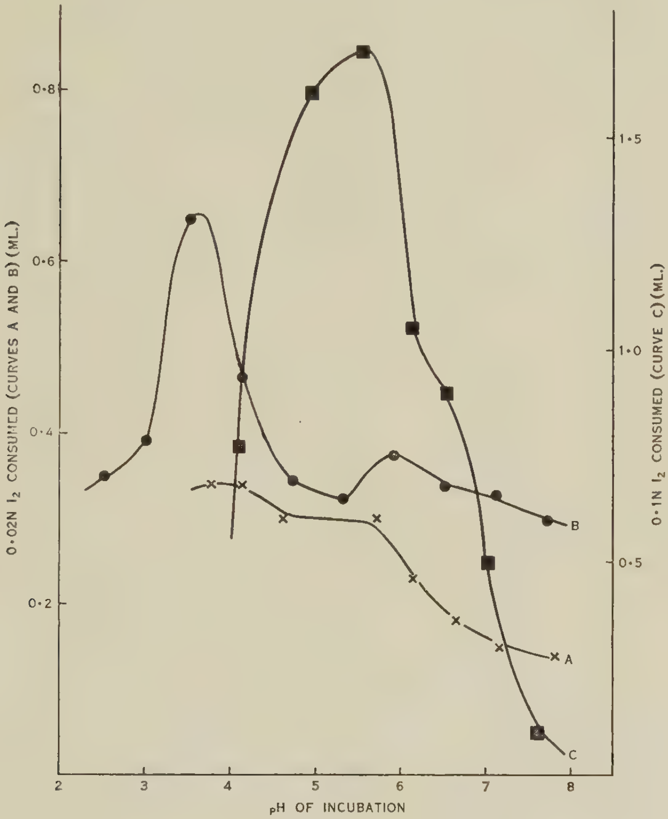

Fig. 3.—pH-activity curves of the *A. oryzae* enzyme using SCMC as substrate in citrate-phosphate buffers at 37°C.

A, SCMC 0.67 per cent., enzyme 0.14 mg./ml., incubated 30 min.

B, SCMC 0.10 per cent., enzyme 0.36 mg./ml., incubated 30 min.

C, SCMC 0.67 per cent., enzyme 10 mg./ml. incubated 6 hr.

The value of approximately 29 per cent. for the maximum breakdown of SCMC seems rather high to agree with the calculated value of 23 per cent., but until less complex enzyme mixtures are available it cannot be decided whether other linkages than those between unsubstituted glucoses are attacked. Kristiansson (1950) suggests that barley malt "cellulase" attacks the linkages between all the glucose residues of hydroxyethylcellulose with equal ease.

(c) *Effects of pH*

The pH-activity curve for the hydrolysis of SCMC is strongly influenced by the concentration of the substrate and the length of the incubation period. Figure 3, curve *B* seems to show the effect of pH on the enzyme activity with the least inactivation and disturbance by colloid effects.

Figure 4 confirms the choice of pH 5.0 as a reasonable average pH at which to measure a series of β-glucosidase activities.

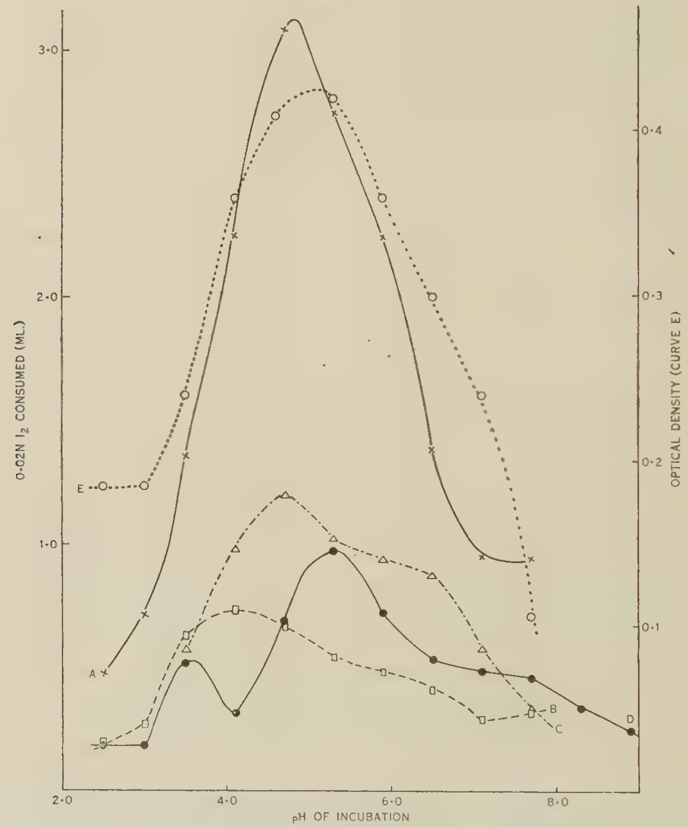

Fig. 4.—pH-activity curves for the *A. oryzae* enzyme acting on various β-glucosidic substrates in citrate-phosphate buffers at 37°C., incubation time 30 min.
A, 0.0013M aesculin, enzyme 0.13 mg./ml.
B, cellodextrin 0.13 per cent., enzyme 0.13 mg./ml.
C, 0.0067M salicin, enzyme 0.13 mg./ml.
D, 0.0067M cellobiose, enzyme 0.13 mg./ml.
E, colorimetric β-glucosidase, standard conditions, enzyme 0.4 mg./ml.

(d) *Effects of Temperature*

Figure 5*B* shows that the basic assumption on which the "short method" is based—that the slope of the curve of iodine consumption against time is nearly

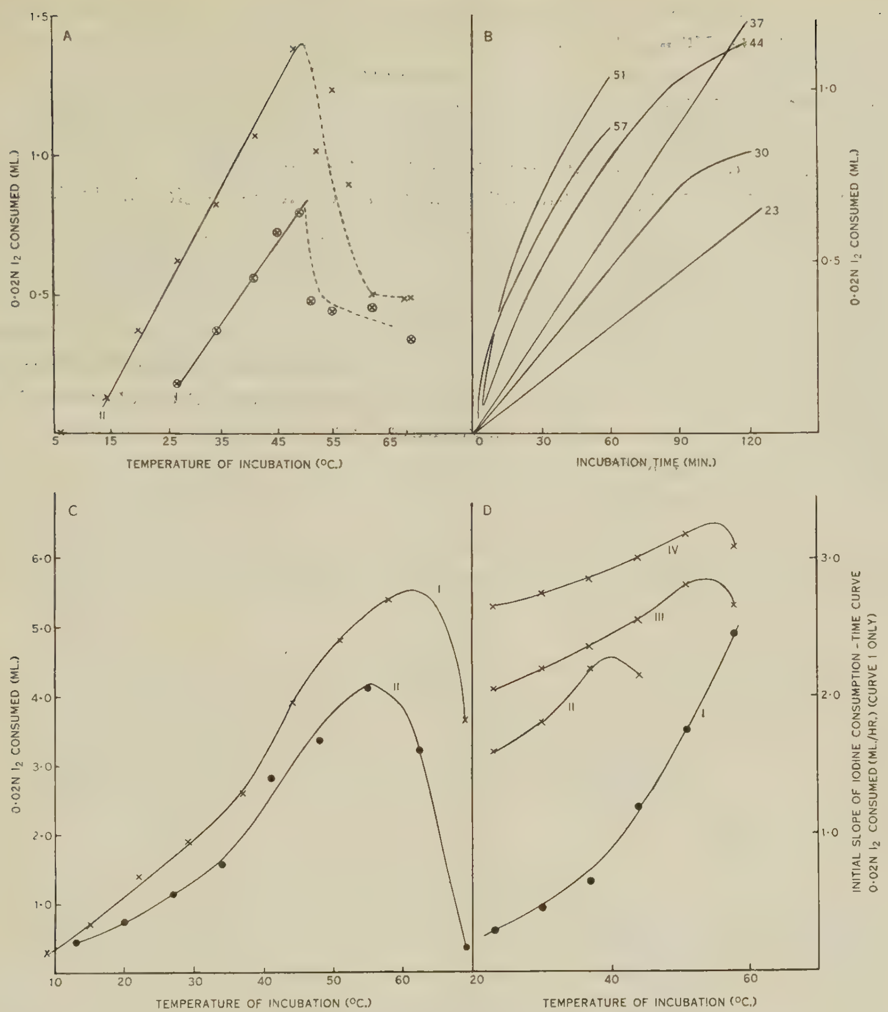

Fig. 5.—Effect of temperature on *A. oryzae* enzymes.

A, activity of the C_x enzyme in decomposing SCMC at various temperatures measured by the short method. I, enzyme 0.2 mg./ml., added in solution. II, enzyme 0.4 mg./ml., added dry.

B, progress of the decomposition of SCMC by the C_x enzyme at various temperatures: SCMC 0.10 per cent., enzyme 0.2 mg./ml. Incubation temperature is indicated in °C.

C, decomposition of cellodextrin and salicin at various temperatures, enzyme 0.4 mg./ml. I, cellodextrin 0.67 per cent. II, 0.0067M salicin.

D, effect of temperature on decomposition of SCMC (from data of Figure 5*B*). I, initial slope of iodine consumption curves plotted against temperature. II, III, IV, iodine consumption after various incubation times plotted against temperature: the curves have been displaced arbitrarily along the abcissa; II, 2 hr. incubation; III, 1 hr.; IV, 0.5 hr.

linear for the first hour or so—is only valid up to about 40-45°C. In conformity with this, in Figure 5*A*, curve I shows a sharp drop in apparent enzyme activity above this temperature to a low and fairly constant level. Since it seemed that this behaviour might be due to inactivation on mixing cold enzyme and hot substrate solutions the method was modified by adding the enzyme as a dry powder to the substrate solution with vigorous mixing. Curve II (Fig. 5*A*), shows relatively higher but still erratic values in the region of 50-55°C.

The method was therefore varied by reducing the final SCMC concentration to a level (0.10 per cent.) where the curve relating substrate concentration to activity (Fig. 7*C*) showed that the effect of substrate concentration on enzyme kinetics was appreciable, adding the enzyme as the dry powder and working according to the long method. None the less, inactivation above 60°C. was too rapid to make it possible to plot the breakdown with time; even at lower temperatures in the region 40-60°C. inactivation was sufficiently rapid to give a marked fall in the apparent optimum temperature as the length of the incubation period was increased.

The plot of $\log_{10}$ (rate of reaction) against the reciprocal of absolute temperature should be linear with a slope of $E/2.303R$ (E = energy of activation,

TABLE 3

ACTIVATION ENERGY OF CERTAIN HYDROLYTIC ENZYMES OF *A. ORYZAE*

Activity	Enzyme Concentration (mg./ml.)	Substrate Concentration	pH (McIlvaine buffer)	Temperature Range (°C.)	Activation Energy (cal./g. mol.)
Hydrolysis of SCMC	1·0	0·10%	5·0	23 - 57	10800
Hydrolysis of cellodextrin	0·20	0·67%	5·0	13 - 54	10250
Hydrolysis of salicin	0·20	0·067M	5·0	15 - 51	9800

R = gas constant). Figure 6 shows that the relationship for three substrates is in fact linear and in Table 3 the calculated energies of activation are given. The differences between the various figures are within the experimental errors of the determination.

(*e*) *Effect of Enzyme Concentration*

When the initial rate of the iodine consumption curve is plotted against enzyme concentration for the decomposition of SCMC, a straight line is obtained as in Figure 7*A*. Hence it is possible, using the long iodometric method and a final SCMC concentration of 0.67 per cent., to estimate the C_x enzyme concentration in a solution of unknown strength. A solution will be said to contain *one unit of* C_x *enzyme per ml.* when under the standard conditions it

gives a curve for the rise in iodine consumption with time, with an initial slope of 0.1 ml. 0.1N I_2/hr. Using the unit so defined the crude enzyme preparation from *A. oryzae* contains 4.9 units/mg.

In Figure 7*B* are shown initial rate plots for cellobiose and cellodextrin done under the same conditions as SCMC; they are both linear, in agreement with normal enzyme behaviour. The plots of iodine consumption in the short method against enzyme concentration for aesculin and salicin were in effect used as calibration curves for measuring enzyme activity against these substrates; the kinetics were not investigated exactly and no explanation will therefore be attempted for the non-linear relation found with salicin.

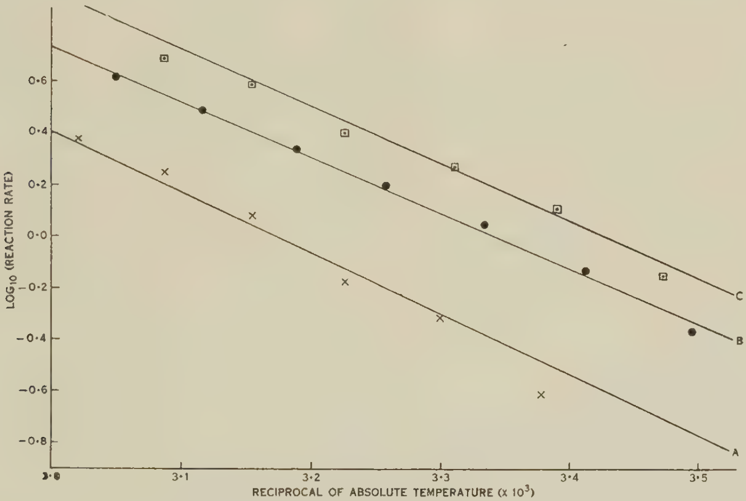

Fig. 6.—Transposition of temperature-activity curves from Figure 5. Substrates: *A*, SCMC; *B*, salicin; *C*, cellodextrin.

(*f*) *Effect of Substrate Concentration*

When an attempt was made to follow the variation of C_x enzyme activity with the concentration of SCMC solution by making up SCMC solutions of relatively high concentration (1.0, 1.5 per cent.) and preparing serial dilutions, it was found that the results obtained for enzyme activity were extremely erratic and depended on the previous history of the solution. In the range 0.15-1.0 per cent. final concentration of SCMC the values for the activity were higher or lower than the initial value and were not reproducible at successive attempts. On standing 3-5 days the activity values tended to rise or fall to a value nearly the same as that of the original undiluted solution. Below 0.15 per cent. final concentration the activity fell away towards zero. The following method was finally found to give reproducible results. SCMC was made up directly as a 0.15 per cent. solution and a series of dilutions prepared. These and the original solution were allowed to stand 3 days and the activity then measured.

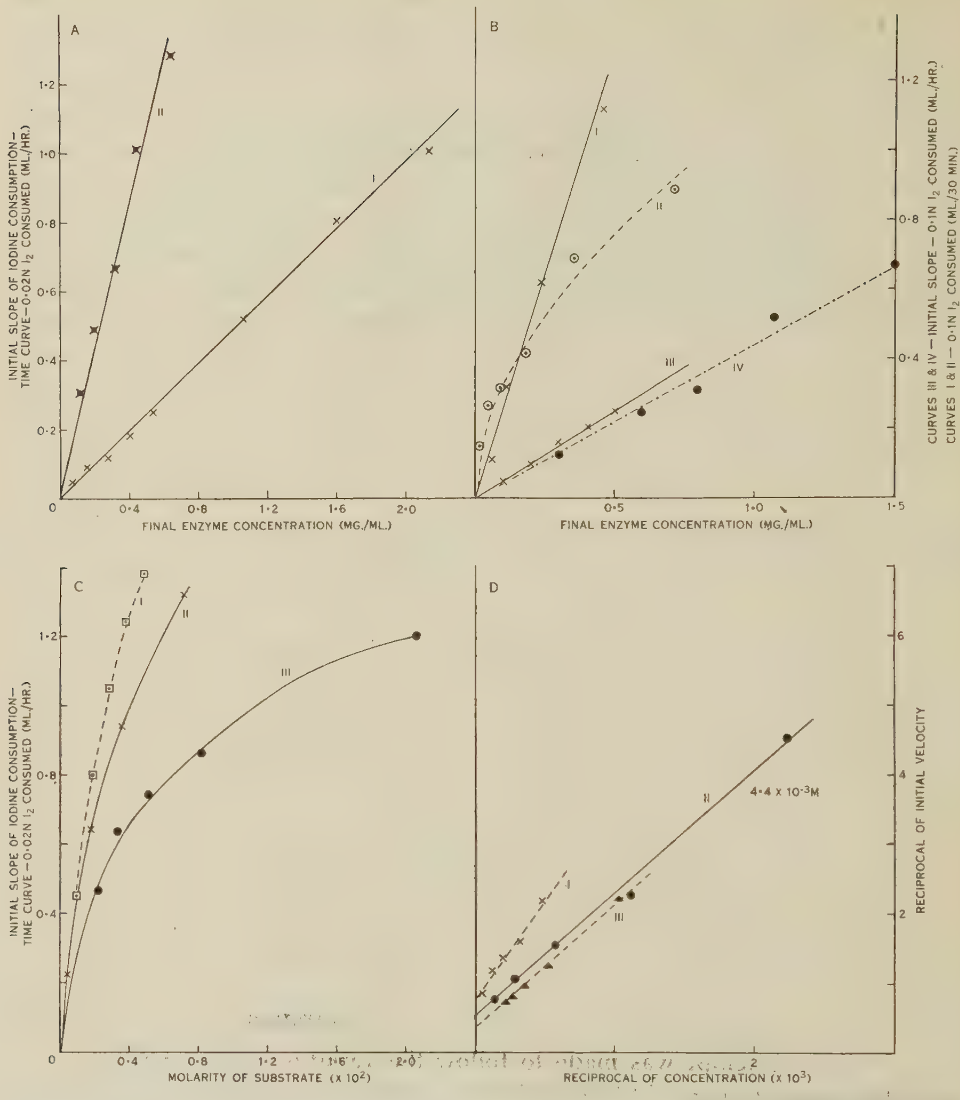

Fig. 7.—Effect of enzyme and substrate concentration on the activity of *A. oryzae* enzymes.

A, enzymic hydrolysis of SCMC at 37°C. I, 0.67 per cent. SCMC, pH 5.5, 0.1N I_2. II, 0.10 per cent. SCMC, pH 5.1, 0.02N I_2.

B, enzymic hydrolysis of various substrates at 37°C. and pH 5.0 at varying enzyme concentrations. I, 0.0013M aesculin. II, 0.0013M salicin. III, 0.0067M cellobiose. IV, 0.33 per cent. cellodextrin.

C, enzymic hydrolysis of various substrates at 37°C. and pH 5.0 at varying substrate concentrations. I, SCMC, enzyme 0.66 mg./ml. II, cellodextrin, enzyme 0.2 mg./ml. III, salicin, enzyme 0.66 mg./ml. SCMC and cellodextrin molarities are those of the calculated glucose equivalents.

D, transposition of Figure 7*C*. I, salicin. II, cellodextrin. III, SCMC.

The results for the hydrolysis of SCMC, salicin, and cellodextrin have been used to determine the Michaelis constants for the three enzymic reactions according to the method of Lineweaver and Burk (1934).

(g) *Inhibition and Activation of the* C_x *Enzyme*

There appears to be very little in the literature on the inhibition of cellulase; the scattered literature on the inhibition of β-glucosidase has been summarized by Veibel (1950, p. 617), known inhibitors being heavy metals, oxidizing agents, formaldehyde, and carbonyl reagents. A few substances are listed by Ziese (1931) as inhibitors of the hydrolysis of hydroxyethyl cellulose by snail cellulase. A systematic preliminary survey under standard conditions was therefore instituted to find what classes of substances inhibit the *A. oryzae* enzyme.

Table 4

MICHAELIS CONSTANTS FOR THE *A. ORYZAE* ENZYMES HYDROLYSING CERTAIN β-GLUCOSIDIC SUBSTRATES (FROM FIGURE 7*D*)

Substrate	Enzyme Concentration (mg./ml.)	Temperature (°C.)	pH (McIlvaine buffer)	K_m (g.-mol/l.)
Salicin	0·066	37	5·0	$3{\cdot}6 \times 10^{-3}$
Cellodextrin (D.P. 100)	0·2	37	5·0	$3{\cdot}2 \times 10^{-3}$
SCMC	0·66	37	5·0	$4{\cdot}4 \times 10^{-3}$

It was found that SCMC solutions prepared by method (i) (dissolution in the cold) gave highly erratic results for the various reagents tried. Solutions made by method (ii) were therefore freshly prepared every 2 days. As a standard procedure the short method for estimating cellulase was employed, the final concentration of crude enzyme being 0.4 mg./ml., at 37°C., and 1 ml. of reagent solution being added to each 15 ml. of the enzymic digest. The iodometric method employed imposed a severe limitation on the concentration of any reagent that itself reacted with iodine or liberated iodine from potassium iodide, and 10^{-3}M was usually the maximum permissible concentration to give a workable blank (titration was with 0.02N reagents throughout). With each series of reagents tested a blank experiment was run with only water added as a check on alteration in properties of the SCMC solution or small changes in enzyme concentration. The velocity found in the presence of the reagent was reported as percentage of the velocity in the blank experiment.

When it was found that 8-hydroxyquinoline alone amongst metal chelating agents had an inhibitory effect on the enzyme, it seemed possible that this might be due to a competition between the chelating agent and the citrate and phosphate ions of the buffer. Acetate buffer (0.1M) of pH 5.0 was therefore used in place of the McIlvaine buffer. The final pH of the mixture under the standard conditions was now 5.1 instead of 5.5 as found with the McIlvaine buffer, but the iodine consumption using SCMC solution freshly prepared by method (ii) was almost identical (1.20 ml. 0.02N iodine).

Table 5

ACTIVITY OF VARIOUS REAGENTS AS ACTIVATORS OR INHIBITORS OF THE ENZYMIC DECOMPOSITION OF SCMC, USING TYPE II SCMC SOLUTIONS AND McILVAINE BUFFER

Reagent Type	Reagent	Subsidiary Effect	Concentration	Percentage of Original Reaction Velocity
Metal ions	Co^{++}		10^{-3}M	119
	Cu^{++}		10^{-3}M	92
	Cd^{++}		10^{-3}M	119
	Cr^{+++}		10^{-3}M	112
	Ni^{++}		10^{-3}M	120
	Zn^{++}		10^{-3}M	108
	Al^{+++}		10^{-3}M	109
	Mn^{++}		10^{-3}M	94
	Fe^{+++}	Oxidizing agent	10^{-3}M	134
	Fe^{+++}		10^{-2}M	115
	Mg^{++}		10^{-3}M	128
	Mg^{++}		10^{-2}M	120
	Cu^{++}		10^{-3}M	129
	Pb^{++}	Thiol reagents	10^{-3}M	103
	Ag^{+}		10^{-3}M	94
Metal complexing reagents	8-Hydroxyquinoline		10^{-3}M	9
	Sodium diethyldithiocarbamate			Decomposed by enzyme
	Nitroso R salt		10^{-3}M	108
	α-Nitroso-β-naphthol		10^{-3}M	96
	Diphenylcarbazide		10^{-3}M	146
	Ferron		10^{-3}M	125
	Cupferron		10^{-3}M	54
	Dimethylglyoxime		10^{-3}M	121
	BAL-intrav.	Thiol	10^{-4}M	129
	Quinalizarin		10^{-4}M	90
	Titan yellow		5×10^{-4}M	106
	Rubeanic acid	Thiol	10^{-4}M	112
	Formaldoxime		10^{-3}M	112
	Rhodizonic acid		10^{-3}M	49
	Salicylaldoxime		10^{-3}M	90
	α-Benzoinoxime		10^{-3}M	137
	Cyanide		10^{-3}M	0
	Thiocyanate		10^{-3}M	103
	Hexametaphosphate		1%	131
Thiol reagents	Iodosobenzoic acid	Oxidizing agent	10^{-3}M	176
	p-Chloromercuribenzoic acid		10^{-3}M	126
—NH_2 reagents	Nitrite			No end point
	Formaldehyde	Reducing agent	10^{-3}M	90
Carbonyl regeants	Hydrazine	Reducing agents	10^{-3}M	124
	Phenylhydrazine		10^{-3}M	146
	Semicarbazide		10^{-3}M	121
	Thiosemicarbazide	Thiol	10^{-3}M	101

TABLE 5 (*Continued*)

Reagent Type	Reagent	Subsidiary Effect	Concentration	Percentage of Original Reaction Velocity
Sugar complexing Agents	Boric acid		10^{-2}M	109
	Boric acid		3×10^{-1}M	121
	Molybdate	Oxidizing agent	10^{-2}M	189
	Tungstate		10^{-2}M	114
	Tartar emetic		10^{-2}M	78
Reducing agents	Bisulphite	Carbonyl reagent	10^{-3}M	78
	Dithionite		10^{-3}M	0
Oxidizing agents	Permanganate		10^{-3}M	400
	Chromate			No end point
	Ferricyanide		10^{-3}M	110
	p-Benzoquinone		10^{-3}M	47
Thiols	Thioglycollate		10^{-3}M	227
	Cystine		10^{-3}M	167
	Cysteine		10^{-3}M	147
	Sulphide		10^{-3}M	0
Growth hormones	β-Naphthoxyacetic acid		10^{-3}M	98
	β-Indole propionic acid		10^{-3}M	62
	β-Indoleacetic acid		10^{-3}M	35
	2, 4-Dichlorophenoxy-acetic acid		10^{-3}M	105
Bases (complex)	Caffeine		10^{-1}M	39
	Adenine		5×10^{-2}M	50
	Quinine		10^{-2}M	40
	Betaine		10^{-1}M	17
	Choline chloride		10^{-1}M	105
Simple amines	Methylamine hydrochloride		10^{-1}M	72
	Dimethylamine hydrochloride		10^{-1}M	51
	Trimethylamine hydrochloride		10^{-1}M	175
	Tetramethyl ammonium bromide		10^{-1}M	104
Miscellaneous	Silicofluoride		10^{-1}M	97
	Fluoride		10^{-2}M	107
	Stearate		3×10^{-4}M	119
	Iodine		10^{-4}M	116
	Azide		10^{-3}M	108
	Arsenate		10^{-3}M	104
	Gluconate		10^{-3}M	80
	Gluconate		10^{-2}M	75
	Galactonate		10^{-3}M	93
	Galactonate		10^{-2}M	107

The data collected for various reagents in McIlvaine buffer are summarized in Table 5. Certain of the classes of reagents tried were suggested by the conclusions of authors working with similar enzyme systems, for instance the inhibition of amylase by plant growth hormones (Volker 1950) and caffeine and other bases (Vincent and Lagreu 1950), and galactosidase inhibition by gluconate and galactonate (Nishizawa 1949). Deviations from the normal reaction rate of ± 10 per cent. or less can be regarded as not significant.

Table 6 records results with the use of acetate buffer. Only certain of the reagents listed in Table 5, which had been found to give marked inhibition or which were useful for comparison purposes, were tested in this medium.

TABLE 6

ACTIVITY OF VARIOUS REAGENTS AS ACTIVATORS OR INHIBITORS OF THE ENZYMIC DECOMPOSITION OF SCMC IN ACETATE BUFFER

SCMC Solution	Reagent	Concentration	Percentage of Original Reaction Velocity
Type I	Phosphate	10^{-3}M	113
		10^{-2}M	147
		3×10^{-2}M	193
	Citrate	10^{-2}M	20
	Oxine	10^{-4}M	120
		10^{-3}M	0
	Ferron	10^{-3}M	160
	(Reaction with same SCMC solution in McIlvaine buffer)		194
Type II	Citrate	10^{-2}M	73
	Phosphate	10^{-2}M	63
	Cyanide	10^{-3}M	46
	Dithionite	10^{-3}M	0
	Cupferron	10^{-3}M	0
	Nitroso R salt	10^{-3}M	66
	Oxine	10^{-4}M	101
		10^{-3}M	90
	Ferron	10^{-3}M	102
	Caffeine	10^{-1}M	56
	Quinine sulphate	10^{-2}M	44
	β-Indolepropionic acid	10^{-3}M	59
	Betaine	10^{-1}M	30

The system acetate buffer plus cupferron was chosen for further study since it gave a reasonably sharp end-point uncomplicated by colour formation and precipitates and also since the results of Albert and Gledhill (1947) show that only three ions — Fe^{++}, Fe^{+++}, and Cu^{++} — react with cupferron under "physiological" conditions. The effect of cupferron concentration on the activity of the C_x enzyme is shown in Figure 8, curve *A*. A set of experiments was therefore set up in which the ability of these three ions to reverse the cupferron inactivation of the enzyme was tested. The results are summarized in Table 7. Reactivation by Fe^{++} and by Fe^{+++} cannot be regarded as indepen-

dent of each other in an aerobic system with oxidizing and reducing enzymes present.

Since ferric iron seemed to be the most effective reactivator of the enzyme in the presence of cupferron, the experiments whose results are plotted in Figure 8, curve *B*, were carried out. These show that the inactivation is substantially reversed at a ratio of three Fe^{+++} ions per cupferron molecule and that additional Fe^{+++} leads to a slight activation in accordance with the data of Table 5.

TABLE 7

REVERSAL OF THE ACTION OF CUPFERRON ON THE C_x ENZYME BY CERTAIN IONS

Experiment	Cupferron Concentration	Concentration of Added Ions	Relative Rate
I	—	—	100
II	10^{-3}M	—	0
III	10^{-3}M	Cu^{++} 3×10^{-3}M	49
IV	10^{-3}M	Fe^{++} 3×10^{-3}M	41
V	10^{-3}M	Fe^{+++} 3×10^{-3}M	90

(*h*) *Inhibition and Activation of Salicinase*

It seemed of interest to compare the pattern of inhibition of a β-glucosidase activity with that observed for the hydrolysis of SCMC. For this pur-

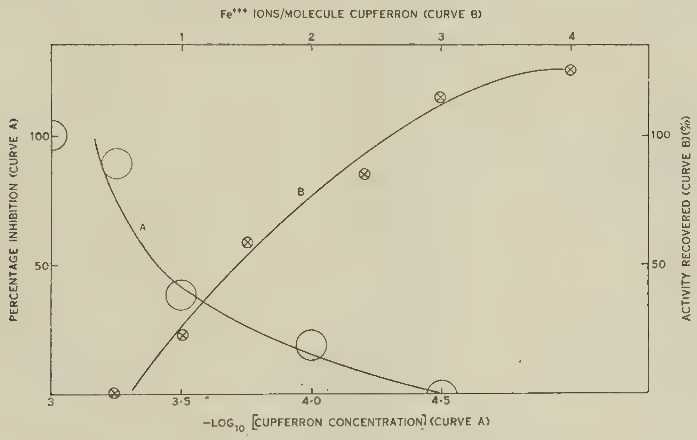

Fig. 8.—Curve *A*; inhibition of C_x enzyme by cupferron; short method conditions; enzyme 0.4 mg./ml., type (ii) SCMC solution.

Curve *B*; reversal of the inhibition by 10^{-3}M cupferron of the C_x enzyme using Fe^{+++}; conditions as in curve *A*.

pose the hydrolysis of salicin was chosen as most convenient and a number of reagents found to have an effect on the SCMC hydrolysis were tested for their effect on this reaction. Since the change from McIlvaine buffer to acetate buffer

appeared to affect the rate of SCMC hydrolysis, a number of buffer systems were investigated to see whether there was any effect on the rate of salicin hydrolysis. The results obtained are shown in Table 8. A variation of ±5 per cent. from the standard probably represents significant activation or inhibition.

V. Discussion

Reese, Siu, and Levinson (1949) have postulated that the C_x enzyme is only produced by fungi in the presence of a β-glucosidic substrate. The existence of a powerful activity in the "mould enzyme" used in this study, which had been secreted into a sucrose-tartrate medium, and the data summarized in Table 1 for *A. oryzae* and the cellulolytic fungus *S. atra* show this conclusion to be untenable. By choosing one fungus and one particular period of incubation it is possible to show that the postulate is true; another set of conditions would seem to prove the exact opposite. The production of C_x enzyme and β-glucosidase by both fungi appears to be adaptive in a general way without showing an absolute dependence on the nature of the substrate. The identity of enzymes produced in the presence and absence of such substrates cannot at present be proved and detailed comparison with the results of other workers will not be attempted.

It will be demonstrated in the next paper that the enzyme activities studied are those of up to eight separate components and that the sums of the varying activities of these components towards different substrates constitute the activities here studied as "C_x enzyme" and "β-glucosidase." This conclusion is borne out by the generally similar properties of the two hypothetical enzymes but is masked by complications arising from the colloidal nature of SCMC. The

Table 8

COMPARATIVE ACTIVITY OF THE *A. ORYZAE* ENZYME IN VARIOUS BUFFERS AND IN THE PRESENCE OF VARIOUS REAGENTS FOR THE HYDROLYSIS OF SALICIN

(*a*) Buffers of pH 5.0

Buffer	Final Concentration	Comparative Activity (McIlvaine buffer = 100)
p-Hydroxyphenylacetate	4×10^{-3}M	121
Ethylenediamine tetra-acetate	10^{-2}M	109
Acetate-HCl	4×10^{-2}M (in acetate)	108
Acetate	10^{-2}M	107
Malate	10^{-2}M	106
Phosphate	10^{-2}M	106
Borate-succinate	10^{-2}M	104
Succinate	2×10^{-3}M	102
Phthalate	10^{-2}M	102
Citrate	10^{-2}M	101
Citrate-phosphate (McIlvaine)		100
Oxalate	10^{-2}M	90
Phenylacetate	4×10^{-3}M	72

TABLE 8 (*Continued*)

(*b*) Reagents in Acetate-HCl Buffer, pH 5.0

Reagent	Concentration	Comparative Activity
Fe^{+++}	10^{-3}M	104
Fe^{++}	10^{-3}M	92
Mg^{++}	10^{-3}M	95
Ca^{++}	10^{-3}M	96
Cu^{++}	10^{-2}M	33
	$3{\cdot}2\times10^{-3}$M	38
	10^{-3}M	62
	$3{\cdot}2\times10^{-4}$M	82
	10^{-4}M	101
Cupferron	10^{-3}M	112
Oxine	10^{-3}M	85
Rhodizonic acid	10^{-3}M	68
Ferron	10^{-3}M	99
Cyanide	10^{-3}M	66
Dithionite	10^{-3}M	98
Bisulphite	10^{-3}M	95
Permanganate	10^{-3}M	108
p-Benzoquinone	10^{-3}M	104
Thioglycollic acid	10^{-3}M	126
Glutathione	10^{-3}M	85
Cysteine	10^{-3}M	92
Sulphide	10^{-3}M	36
p-Chloromercuribenzoic acid	10^{-3}M	85
Quinine	10^{-2}M	83
Caffeine	10^{-1}M	97
Betaine	5×10^{-2}M	103
Semicarbazide	10^{-3}M	90
Phenylhydrazine	10^{-3}M	88
Benzoic acid	10^{-3}M	96
Salicylic acid	10^{-3}M	99
Naphthoxyacetic acid	10^{-3}M	98
2, 4-Dichlorophenoxyacetic acid	10^{-3}M	103
Benzilic acid	10^{-3}M	100
Benzylmalonic acid	10^{-3}M	99
p-Hydroxybenzoic acid	10^{-3}M	102
p-Hydroxyphenylacetic acid	10^{-3}M	92
β-Indolylpropionic acid	10^{-3}M	86
	4×10^{-3}M	114
Phenoxyacetic acid	10^{-3}M	100
Benzyl alcohol	10^{-2}M	97

properties of this substrate appear to set a limit to the types of experiment that can usefully be performed. Kagawa and Katsuura (1951) have shown that the apparent hydrogen ion concentration of solutions of SCMC may be very different from the hydrogen ion concentration of the colloid phase and that it is this latter concentration that determines the behaviour of the SCMC micelles and hence bulk properties of the solution such as viscosity. This type

of behaviour would explain the smearing out of the maxima of the pH-activity curve in concentrated SCMC solution since the pH of the environment in which the enzyme acts will be only qualitatively related to the measured pH of the solution.

The linear activity-temperature relationship in concentrated SCMC solutions and the indifference of the activity to changes in substrate concentration suggest that diffusion is the rate-limiting process, as might be expected in a solution containing large colloidal aggregates. Dürig and Banderet (1950) have shown that the behaviour of SCMC solutions is determined largely by the degree to which they are composed of amorphous and semi-crystalline fractions. The amorphous fractions form a stable colloid sol under all conditions but the semi-crystalline fraction slowly deposits from 1 or 2 per cent. solution as a gel and consists of large aggregates when dispersed. In agreement with these results it was found that high-speed centrifuging of aged solutions of the SCMC used in this study resulted in the deposition of a transparent gel. The observation that many characteristics of the enzyme attack on SCMC solutions depend on the concentration and previous history of the solutions is in harmony with the expected behaviour of such a complex colloid mixture.

It may therefore be concluded that, although the linear relationship between enzyme concentration and activity in concentrated SCMC solutions provides a valuable tool for measuring enzyme concentration, the general properties of these solutions make them unsuitable for detailed investigation of the kinetics of enzymes that hydrolyse polymeric β-glucosidic linkages. If these disturbing effects are disregarded no evidence has been discovered that SCMC is hydrolysed by an enzyme intrinsically different from other β-glucosidases. The kinetic constants (Michaelis constant, energy of activation) are consistent with the unitary hypothesis; however, if they are in fact averaged figures for a group of enzymes no useful argument can be drawn from them. None of the enzyme preparations is yet pure enough to bring departures from the kinetics of single-enzyme behaviour (e.g. non-linearity of Lineweaver-Burk plots) within the limits of the experimental errors.

The comparative results of experiments on the effect of various reagents on the activity of "salicinase" and "cellulase" are also consistent with the hypothesis that each observed activity is the sum of the activities of a set of enzymes hydrolysing β-glucosidic linkages but of different specificity towards the two substrates. The effect of copper on salicinase, which does not go beyond about 65 per cent. inhibition at relatively high concentrations of Cu^{++}, is a valuable indication of the existence of more than one component in this system. In general the two enzyme activities tend to be affected by the same groups of substances, but the degree to which one is affected by a certain concentration of reagent and even whether the reagent will act as an inhibitor or activator cannot be predicted from the behaviour of the other. Thus both enzymic activities seem to be affected by substances of the general formula $Ar(CH_2)_nCOOH$ and this effect seems to be specific, judged by the ineffectiveness of various plant hormone and saligenin analogues, but the amount and sign of this effect varies with the substrate and the nature and concentration of the reagent.

It is of interest to note that the pattern of inhibitors active against "salicinase" and "cellulase" is in agreement neither with the list given by Veibel (1950) for β-glucosidases nor with the results of Ziese (1931) for the action of malt and snail cellulases on hydroxyethyl cellulose. Ziese found these cellulases to be unaffected by papain or copper ions, and inactivated by cysteine, glutathione, and ferric chloride. This disagreement is another demonstration of the fact that "cellulase" systems from different organisms cannot be comparable and that a number of different mechanisms may well be operative.

For inhibitors and activators of the hydrolysis of SCMC the whole picture is obviously complicated by the nature of the buffer, the history of the SCMC solution, and the interaction of buffer and SCMC. If it is postulated that the ability of a complexing agent to inhibit the C_x enzyme is conditioned (*a*) by its ability to form a stable complex with the metal activating agent at the pH used, and (*b*) by its ability to form such a complex in competition with the complexing power of buffer and SCMC, most of the results with the heavy metal complexing agents can be interpreted. Only reagents known to give complexes with metals under "physiological conditions"—8-hydroxyquinoline, cupferron, Nitroso R salt (Albert and Gledhill 1947)—inhibit the enzyme.

None the less the data of Table 7 and Figure 8 cannot be unequivocally interpreted as the inhibition of an enzyme activated by Fe^{+++} (or some other heavy metal ion) and the reversal of this inactivation by excess Fe^{+++}. Since these observations cannot be readily harmonized with those on the inhibition of salicinase, it is quite possible that the primary effect of the heavy metal ion may be on the SCMC rather than the enzyme. Alternatively, cupferron may be the primary inhibitor whose influence is reversed by complexing with Fe^{+++}. The difference between the behaviour of chelating agents and that of more specific inhibitors with change of buffer is hard to reconcile with this hypothesis, and the action of oxidizing, reducing, and certain other reagents is consistent with an activation by Fe^{+++}.

Only experiments with purified enzymes and substrates can give any certain results in this type of investigation and the complex patterns of activity are what might be expected from a crude mixture of enzymes. It is impossible therefore to draw any but the most general conclusions.

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FUNGAL CELLULASES

II. THE COMPLEXITY OF ENZYMES FROM *ASPERGILLUS ORYZAE* THAT SPLIT β-GLUCOSIDIC LINKAGES, AND THEIR PARTIAL SEPARATION

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Summary

Filter paper electrophoresis and paper chromatography have been used to test the homogeneity of the β-glucosidase of *Aspergillus oryzae*. In the crude enzyme preparation there are at least eight components capable of breaking β-glucosidic linkages and showing varying degrees of specificity towards different substrates. These are active not only in splitting simple glucosides but also in depolymerizing sodium carboxymethyl cellulose. The only exception is provided by the enzymes splitting *p*-nitrophenyl-β-glucoside, which are sharply limited to two closely related components. This two-component mixture has been partially purified from accompanying enzymes.

Neither the postulate of a single β-glucosidase nor that of a specific enzyme for each substrate will fit the experimental results. It also appears that the concept of an enzyme (C_x) specific for polymeric β-glucosides and qualitatively different from other β-glucosidases must be abandoned in this instance.

Attempts to separate and purify various enzymes have given results in harmony with the hypothesis of multiple enzyme components.

I. INTRODUCTION

The most recent review of the β-glucosidases is that of Veibel (1950). From 1920 onwards the primary concern of most workers in this field has been the specificity of the enzyme towards different aglycones and the effects of most possible variations in the nature of the aglycone have been investigated. The primary assumption made is that the variations in activity with variation of aglycone are those of a single enzyme, differing according to source, for instance almond emulsin, yeast emulsin, and animal β-glucosidase, but homogeneous from any given source. Veibel states (p. 593), "Originally the concept was that each naturally occurring glycoside required a special enzyme, e.g. amygdalase, salicinase, arbutinase. However, as the artificially prepared glycosides proved to be hydrolysable under the influence of such enzymes as emulsin or invertase, it became clear that the specificity of the enzymes was not as great as at first presumed, but that on the other hand, these preparations were not single enzymes but mixtures of different glycosidases. Further experiments led to the assumption that all β-glucosides are hydrolysed by one enzyme, a β-glucosidase."

In general, proof of the homogeneity of the β-glucosidase used in various studies has been scanty or lacking, and purification and characterization of

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the enzyme itself has been subordinated to the interest in the effect of variation in aglycone on the activity of the enzyme. There seems to be no account of the thorough investigation by all available techniques of the homogeneity of any sample of β-glucosidase. Miwa and Tanaka (1949) have shown that in apricot emulsin the β-glucosidase, β-xylosidase, and β-galactosidase activities, which are often considered by members of the Helferich school to be activities of a single enzyme, can in fact be partially separated by precipitation with ammonium sulphate or methanol, and by absorption on aluminium hydroxide. The nature of the evidence presented—change in the ratio of the activities in fractions prepared by various treatments—is very similar to that presented in this paper, and electrophoresis of such preparations might give similar results. Veibel himself states (p. 588), "Further investigation of the effect of electrophoresis should, however, be of value, as not all the possibilities of this efficient method of separation of (even related) proteins are exhausted by the experiments mentioned."

Pigman (1943) also came to the conclusion that the wide range of specificity of various β-glucosidases could not be explained on the basis of a single enzyme and postulated a class of closely related enzymes all showing specific ability to hydrolyse β-glucosidic linkages.

Any check on the homogeneity of the β-glucosidase present in the *A. oryzae* preparation would also serve as a check on the relationship between the β-glucosidase and the C_x enzyme since these enzyme activities would be expected to segregate independently if they were due to unrelated components of the mixture.

II. Methods

The methods used for determining enzymic activities have been outlined in the preceding paper. Interfering substances present in the crude enzyme material prevented the use of spectrophotometric methods or nitrogen determinations for estimating protein concentrations; a modification of the colorimetric procedure of Stiff (1949) in which very small amounts of protein are estimated through the copper taken up in the biuret reaction was therefore used.

Into each of nine 15 ml. centrifuge tubes were pipetted 8.0 ml. of 5 per cent. trisodium phosphate solution, and 200 mg. of copper phosphate were added to each tube except No. 2. Water and protein solution were added to the tubes according to following schedule:

Tube	1	2	3	4	5	6	7	8	9
Water (ml.)	2·0	0	1·7	1·4	1·1	0·8	0·5	0·2	0
Protein solution (ml.)	0	2·0	0·3	0·6	0·9	1·2	1·5	1·8	2·0

All tubes except No. 2 were now stoppered and shaken for 90 min. in a shaker, then centrifuged and the supernatant filtered off through an open paper (Whatman 541) to remove any floating copper phosphate. From 1.0 to 8.0 ml. of the filtrate (according to the amount of copper expected) was added to 2.0 ml. of 0.5 per cent. sodium diethyldithiocarbamate solution and the whole made up to 20 ml. The colour developed was measured at 440 mμ in a Coleman

spectrophotometer. The amount of copper taken up by the protein in tubes 3-9 could now be calculated and plotted against volume of protein solution. A least-squares line was then drawn to fit the points. The slope of this line (μg. copper dissolved per ml. of protein solution) was reproducible to less than 5 per cent., in spite of erratic variations ($\pm$25 per cent.) in single points.

A series of experiments using certain pure proteins in solutions of known strength showed the following ratios for wt. of protein/wt. of copper: haemoglobin 7.1; trypsin 7.2; pepsin 7.3; bovine serum albumin 7.6; giving a mean (7.3) close to the value (7.1) reported by Stiff. The weight of copper dissolved by 1 ml. of protein solution was therefore multiplied by 7.3 to give the weight of protein in 1 ml. of solution. Figure 1 shows some typical results.

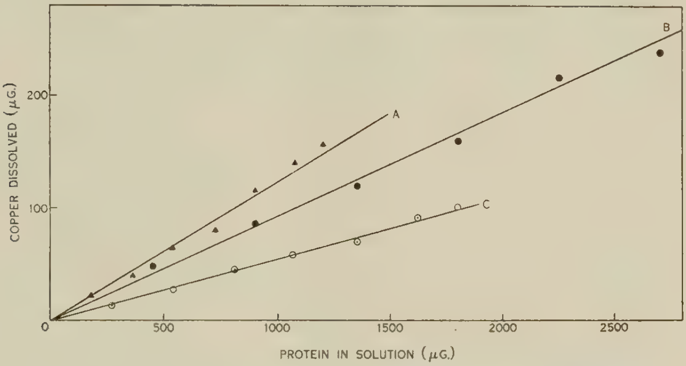

Fig. 1.—Microbiuret estimation of proteins. *A*, haemoglobin; *B*, partially purified *A. oryzae* enzyme (estimated to contain 76 per cent. protein); *C*, crude mould enzyme (estimated to contain 34 per cent. protein). No allowance has been made for the moisture content of the air-dry protein.

The limits of the method are about 1-10 mg. of protein dissolved in a minimum of 11 ml. of H_2O. Its usefulness thus lies in the direct estimation of dilute solutions of protein rather than in the assay of extremely small amounts.

III. Purification of Enzymes

(*a*) *Concentration of* p-*Nitrophenyl-β-glucosidase*

Crude mould enzyme (20 g.) dissolved initially in 750 ml. of pH 7 phosphate buffer was fractionated with ammonium sulphate and the fraction precipitating between 55 and 65 per cent. saturation was retained. This material was refractionated at volumes of 250 and 100 ml. The final material was dissolved in 400 ml. of pH 5 acetate buffer, 500 ml. of 3 per cent. calcium phosphate gel added, and the pellet from centrifugation extracted with three 100 ml. lots of 0.067M disodium phosphate. The extract was adjusted to pH 7 with solid monosodium phosphate, and an equal volume of pH 5 acetate buffer added, followed by 180 ml. of 3 per cent. calcium phosphate gel. The pellet

was extracted with three 30 ml. lots of 0.067M Na_2HPO_4, the extract adjusted to pH 7, dialysed in cellophane overnight against distilled water and the final solution freeze dried. Of the 6.6 g. of original enzyme protein 86 mg. (1.3 per cent.) were recovered as a white powder containing 56 per cent. of protein and 2.8 per cent. of carbohydrate.

TABLE 1

DISTRIBUTION OF VARIOUS ENZYME ACTIVITIES AT DIFFERENT STAGES OF THE CONCENTRATION OF THE *p*-NITROPHENYL-β-GLUCOSIDASE ACTIVITY IN *A. ORYZAE*

Enzyme	After Ammonium Sulphate Fractionation		After Calcium Phosphate Absorption		After Dialysis		
	Activity Recovered (%)	Purification Factor	Activity Recovered (%)	Purification Factor	Activity Recovered (%)	Purification Factor	Loss on Dialysis (%)
Colorimetric glucosidase	23	2·9	19·6	12·7	12·7	9·8	35
Salicinase					14·9	11·5	
C_x enzyme	15	1·9	4·9	3·1	2·2	1·7	55
Cellobiase					2·0	1·5	
Amylase					1·3	1·0	
Sucrase					0·8	0·6	
Esterase					0·13	0·09	
Viscometric protease	1·2	0·15			0·08	0·06	
Recovered protein	7·9		1·54		1·30		16

The inactivation of certain of the enzymes on dialysis forms a serious barrier to their isolation by methods involving solutions of salts. The C_x activity was not absorbed on shaking with cellulose powder at pH values from 5 to 8 for a few minutes and the activity lost on dialysis was not restored by calcium, zinc, manganese, magnesium, or iron ions. The 16 per cent. loss of protein on dialysis suggests that the enzymes may themselves be slowly dialysable through cellophane, although other possibilities, e.g. slow proteolysis by residual protease, cannot be excluded.

Filter paper electrophoresis of the purified enzyme showed that the bulk of the protein material was concentrated in a single spot coinciding in position with that of the *p*-nitrophenyl-β-glucosidase. Determination of other enzyme activities (see next section) showed that the small amounts of protein in other parts of the paper still contained appreciable enzyme activities. The colorimetric β-glucosidase was still inhomogeneous by paper chromatography, showing the same distribution of the two components as the original crude preparation.

Attempts to concentrate or fractionate other enzyme activities by the usual methods (solvent and salt precipitation or adsorption) gave erratic results. The only experiment giving an unequivocal result is described below.

(*b*) *A Demonstration of the Non-Homogeneity of the* C_x *Enzyme*

The C_x activity remained in solution when ethanol was added to a 1 per cent. solution of the crude enzyme to give a final ethanol concentration of 40 per cent. (v/v), and it was found that ammonium nitrate gave satisfactory results as a protein precipitant in this medium. The experiments were carried out at 10°C., the C_x activity being stable for some hours in 40 per cent. ethanol at pH 5.5 at this temperature. The solutions were equilibrated 1 hr. after the ammonium nitrate had been dissolved, the precipitate centrifuged down, and C_x and viscometric protease activities determined in the supernatant. The results are shown in Figure 2 and demonstrate the probable presence of at least two components in the C_x enzyme.

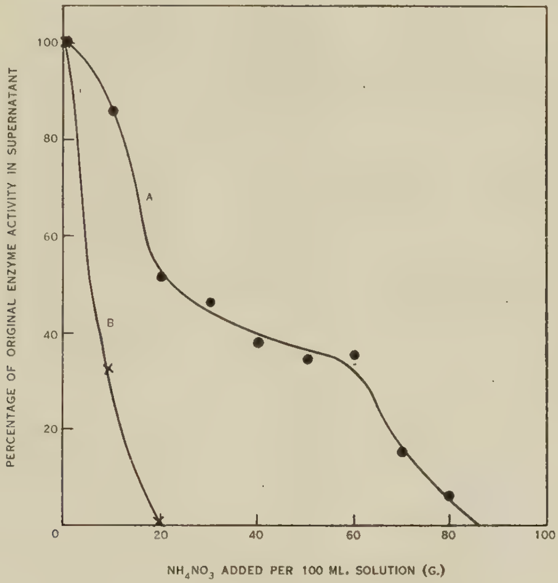

Fig. 2.—Precipitation of enzymes by ammonium nitrate in 40 per cent. ethanol. *A*, C_x enzyme; *B*, viscometric protease.

(*c*) *Activity of* C_x *and β-glucosidase in Various Enzyme Preparations*

It will be shown in the next section that precipitation by 40 per cent. ethanol under certain conditions and passing enzyme solutions through an ion-exchange column lead to differential separation of two components of the *p*-nitrophenyl-β-glucosidase. Material prepared by these methods was tested for activity against a number of substrates and compared with the original crude enzyme and a specimen purified by electrodialysis. The protein concentration of the test solutions was proportional to the amounts of the original protein material retained in each preparation. The relative activities (cellulase = 1) for a given enzyme preparation are the ratios of the number of β-glucosidic linkages split per hour under the standard conditions for any substrate to the number split in SCMC. The results are summarized in Table 2.

IV. Filter-paper Electrophoresis and Chromatography

(a) *Filter-paper Electrophoresis*

The method of filter-paper electrophoresis provides a rapid and convenient method of investigating enzyme homogeneity (Cremer and Tiselius 1950). The application of this method to the crude enzyme mixture from *A. oryzae* will form part of a forthcoming paper by Gillespie and Woods. Some indication of the type of results obtained working with the crude *A. oryzae* enzyme has already been given by Gillespie, Jermyn, and Woods (1952). The same standard conditions were employed in all filter-paper electrophoreses (Whatman 3MM paper, veronal buffer of ionic strength 0.025 and pH 8.6, 6 hr. at a potential gradient of 9 V./cm.). Both the crude enzyme and the *p*-nitrophenyl-β-

Table 2

COMPARISON OF THE ACTIVITIES OF VARIOUS ENZYME PREPARATIONS AGAINST DIFFERENT β-GLUCOSIDIC SUBSTRATES

Substrate	Crude Enzyme		Ethanol-Precipitated Enzyme		Enzyme Prepared by Passing Through Ion Exchange Column		Enzyme Prepared by Electrodialysis	
	Activity Recovered (%)	Relative Activity $C_x = 1$	Activity Recovered (%)	Relative Activity $C_x = 1$	Activity Recovered (%)	Relative Activity $C_x = 1$	Activity Recovered (%)	Relative Activity $C_x = 1$
Cellobiose	100	4·1	97	8·1	107	6·9	98	6·3
Salicin	100	2·0	25	1·0	27	0·9	60	1·9
Aesculin	100	6·1	65	8·1	89	8·4	78	7·5
Methyl β-glucoside	100	0·11	38	0·08	70	0·09	70	0·11
SCMC	100	1·0	49	1·0	64	1·0	64	1·0
p-Nitrophenyl-β-glucoside	100	0·2	160	0·6				

glucosidase concentrate were tested for the distribution of the activity against various substrates along the paper. The results are plotted in the form of histograms in Figure 3. The determination of cellobiase was not attempted since it was judged that the activities to be measured were too low to ensure reasonable activity by the available technique.

The degree of variability between different runs is illustrated by the three different results for the "colorimetric β-glucosidase"; the difference between peaks of activity in different runs judged to be due to the same component was not more than 2 cm. Woods (personal communication) finds about the same degree of variability with other enzymes which he has tested; there are too

many small possible sources of variation to expect absolute reproducibility in shape and position of peaks of enzyme activity, and personal judgment enters to some extent into the comparison of the various histograms. No component has been postulated unless a corresponding peak appears on at least two histograms. The existence of only one component actively hydrolysing p-nitrophenyl-β-glucoside could be checked by observing that a single well-defined yellow spot appeared after the paper had been sprayed with a solution of the glucoside in pH 7.5 phosphate buffer and incubated at room temperature.

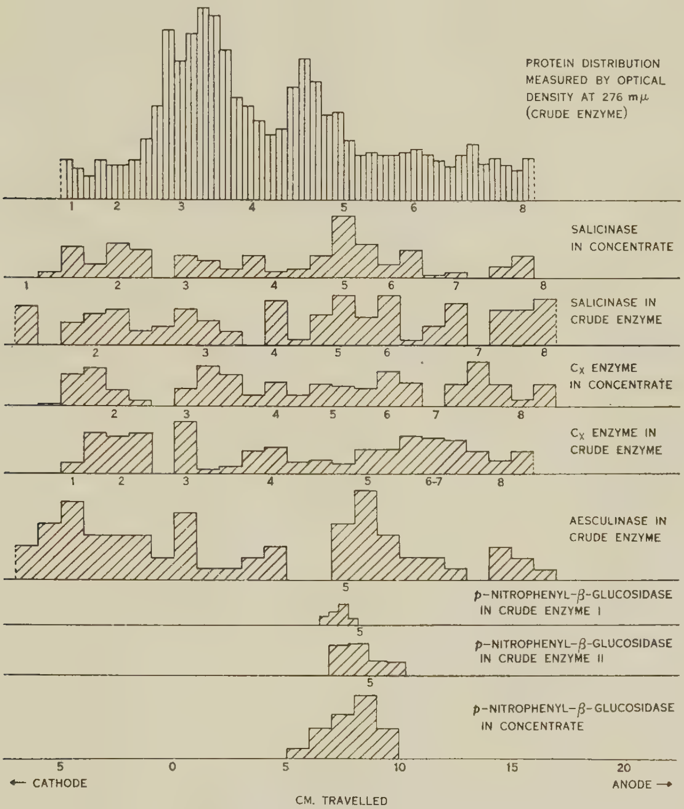

Fig. 3.—Distribution of various enzyme activities of *A. oryzae* enzyme preparations after filter-paper electrophoresis at 9 V./cm. for 6 hr. at pH 8.6 (concentrate = semi-purified p-nitrophenyl-β-glucosidase). Numbers are assigned to what are believed to be identical components.

(*b*) *Filter-paper Chromatography*

The technique used has already been described (Gillespie, Jermyn, and Woods 1952), and a full investigation of the paper chromatography of the *A. oryzae* enzymes will be made the subject of a later paper. The chromatographic techniques used were based on those of Reid (1950).

The p-nitrophenyl-β-glucosidase appears as a single component (No. 5) on filter-paper electrophoresis. Nevertheless, if a solution is spotted on a paper chromatogram, developed using 0.01M pH 5.8 McIlvaine buffer and acetone (60-40 v/v), and the dried paper sprayed with an 0.05 per cent. solution of p-nitrophenyl-β-glucoside in 0.2M phosphate buffer at pH 7.5, the presence of two components is revealed by yellow areas against a white background. Component A is a well-defined round spot of R_F 0.7 after 20 cm. solvent travel and component B a long streak of R_F 0.2-0.55. Both the crude enzyme and the concentrate gave the same pattern. Almond emulsin showed two similar components and a third immobile component. A large number of enzyme preparations were available in this laboratory from various attempts to purify enzyme components. On testing these preparations it was found that one prepared by passing a solution of the crude enzyme through a mixed-bed ion-exchange column was lacking in component B, while one prepared from the supernatant after a precipitation with ethanol (final conditions 40 per cent. ethanol, —5°C., and pH 8) was lacking in component A. Component 5 is thus further divisible into the two components 5A and 5B, both of which travel into the same position when investigated by filter-paper electrophoresis.

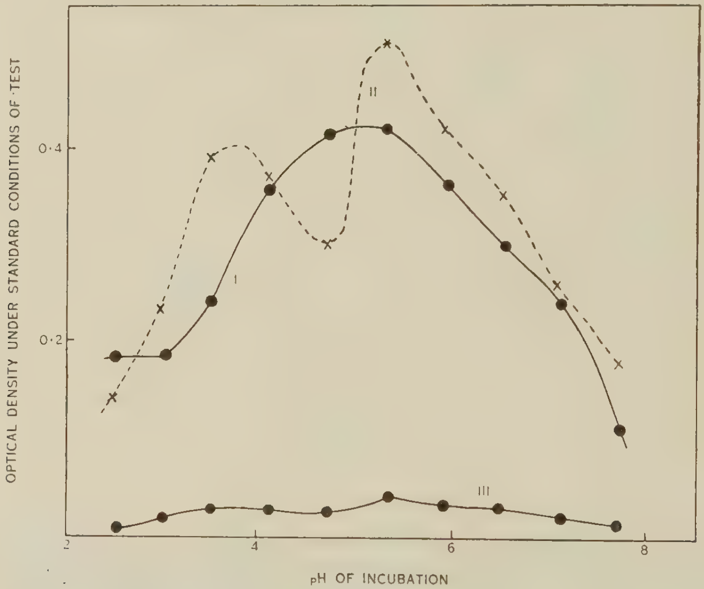

Fig. 4.—pH-activity curves for the p-nitrophenyl-β-glucosidase. I, crude enzyme. II, enzyme passed through ion-exchange column. III, curve II reduced in scale for comparison with curve I at equivalent protein concentrations.

Component 5A was further investigated by examining its pH-activity curve. The results are shown in Figure 4.

It can be seen that component 5A is a very minor fraction of the original component 5. Yet the pH activity curve shows a pronounced double peak and it seems almost certain that further fractionation could again resolve it into

two components $5A_1$ and $5A_2$. The appearance of more than one peak in the pH-activity curves for the *A. oryzae* enzyme acting on various β-glucosidic substrates appears to be quite common (cf. first paper of this series) and is a further argument for the heterogeneity of the enzyme systems involved.

Almond emulsin, which shows a somewhat similar picture to the *A. oryzae* enzyme on chromatography, was subjected to filter paper electrophoresis under the standard conditions. *p*-Nitrophenyl-β-glucosidase appeared as a streak stretching between points 0.5 and 9.0 cm. towards the cathode and showing no signs of resolution into components. Salicinase, which was detected by spraying with salicin solution, incubating, spraying with a solution of *p*-anisidine hydrochloride in ethanol, and heating to develop the colour, appeared as a streak stretching from a point 2 cm. towards the anode to one 6 cm. towards the cathode. Almond emulsin thus does not contain the same β-glucosidase components as the *A. oryzae* enzyme, but may none the less contain a system of components fully as complex.

V. Discussion

Veibel, in the statement quoted in Section I, admits two alternatives — either a battery of β-glucosidases each with its own substrate, or a single β-glucosidase with a wide range of specificity towards various substrates. The present work shows neither of these alternatives to be true for enzymes from *A. oryzae,* where there are a number of β-glucosidases, none of which has absolute specificity for any one substrate. It is evident that these β-glucosidases of *A. oryzae* form a group of proteins of very closely related properties, and that no statement on the specificity of the crude "β-glucosidase" produced by this mould has any real meaning.

It is also evident that the complexity of the material currently available in this laboratory, and the small relative amounts of each component present, make the problem of isolating any one of them and examining its properties almost insurmountable. The mould would have to be grown under conditions leading to maximum β-glucosidase production with as little contaminating protein as possible before any useful attempts could be made in this direction. Even so, the example of *p*-nitrophenyl-β-glucosidase shows the difficulty of proving homogeneity. It appears from the few rough tests made that almond emulsin is also complex, and that statements on its specificity must be viewed with reserve.

Gillespie and Woods (unpublished data) have shown that certain of the enzymes of *A. oryzae,* which appear to be separated into more than one component by filter paper electrophoresis, can actually be fractionated to give preparations showing a single component corresponding to one of the multiple components of the crude enzyme. It must be admitted that the failure to effect such resolution with β-glucosidases makes it possible that the observed components may be artefacts in spite of all evidence to the contrary. Final justification of the conclusions drawn in this paper must therefore await such a resolution in this or a closely related system.

The second conclusion that can be drawn from these experiments also conflicts with many of the conclusions drawn from work carried out with other enzyme systems. It is that in the *A. oryzae* system the enzymes breaking down monomeric β-glucosides do not differ qualitatively from those breaking down polymeric β-glucosides. There is thus no need to assume the existence of a "cellulase" or a "C_x enzyme" to explain the breakdown of long-chain cellulose derivatives in solution. The electrophoretic evidence for the identity of the enzymes capable of breaking down SCMC and β-glucosides is confirmed by the identical values within the experimental error of the observed Michaelis constants and energies of activation of the enzymes when hydrolysing salicin, SCMC, and cellodextrin. Although the evidence is by no means complete at this stage, it is strong enough to suggest that the existence of enzymes of the "C_x" type as a separate species is open to doubt. Variation in specificity towards different substrates among enzymes from different sources is to be expected and cases have been cited by Levinson, Mandels, and Reese (1951) in which near absence of salicinase and cellobiase activity accompanies high C_x activity in fungal culture filtrates. These may represent no more than one extreme in the possible range of specificities, and until the C_x enzyme has been shown in some case to act by a radically different mechanism there seems no justification for removing it from the general class of β-glucosidases. In any case it seems at present most unwise to generalize about the mechanism of "cellulase" action on the results of a set of experiments using enzymes derived from any single organism.

The enzymes splitting *p*-nitrophenyl-β-glucoside appear to be part of a separate system which may not be closely related to the other β-glucosidases. The segregation of the various β-glucosidases together in the purification of *p*-nitrophenyl-β-glucosidase may be no more than a reflection of the fact that carbohydrases form a group of proteins more closely related in properties than the other enzymes. This is in agreement with the work of Niwa (1943) who found that the β-glucosidases from animal viscera splitting *p*-nitrophenyl-β-glucoside were considerably different in their properties from those splitting salicin and phenyl-β-glucoside. The easily applied colorimetric method for β-glucosidase is thus a completely unreliable index of total enzyme activity.

VI. Acknowledgments

The author is deeply indebted to Mrs. M. C. Wilkinson for her able technical assistance. He also wishes to thank Mr. J. M. Gillespie for preparing the *A. oryzae* enzyme concentrates and the electro-dialysed and exchange resin de-ionized enzyme preparation used in these investigations, Mr. E. F. Woods for carrying out filter paper electrophoreses, and Mrs. H. M. Forss for certain enzyme determinations.

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STUDIES ON THE DIGESTION OF WOOL BY INSECTS

VII. SOME FEATURES OF DIGESTION IN THREE SPECIES OF DERMESTID LARVAE AND A COMPARISON WITH *TINEOLA* LARVAE

By D. F. WATERHOUSE*

[*Manuscript received May* 30, 1952]

Summary

Three species of dermestid larvae (*Anthrenocerus australis, Anthrenus verbasci,* and *Attagenus piceus*) are shown to be capable of digesting wool.

The midgut of these species is simple, without differentiation into zones, and the epithelium consists of simple columnar cells, together with nidi of regenerative cells. A peritrophic membrane is present. The midgut of *australis* and *verbasci* is virtually devoid of tracheae, and although the midgut of *piceus* is better supplied, its tracheation is comparable with that of *Tineola,* which is poorly tracheated compared with many other insects.

Examination under polarized light of wool in the digestive tract shows that many fibres are disintegrated as they pass down the gut.

The pH of the midgut approximates 7.0 and the oxidation-reduction potential falls in the range — 190 to — 230 mV.

The highly reducing conditions in the dermestid midgut reduce the disulphide bonds of wool keratin, permitting attack by proteolytic enzymes. Most of the cysteine thus produced is not degraded further and is excreted. Dermestid larvae, therefore, only under exceptional circumstances produce metal sulphides after ingestion of appropriate salts. The faeces remain of normal colour except when a coloured cysteine-metal complex (Co) or reduction product (Te) is formed. By contrast, in *Tineola* larvae, portion of the cystine is degraded further by a process that appears to be partly chemical (high pH) and partly enzymic (a desulphydrase, capable of splitting off H_2S).

Available evidence indicates that neither dermestid nor *Tineola* larvae are capable of digesting the water-insoluble fraction (fibroin and sericin C) that forms the bulk of the silk fibre.

I. INTRODUCTION

A considerable amount is now known of the processes whereby wool is digested by larvae of the clothes moth *Tineola bisselliella* (Day 1951*a*, 1951*b*; Linderstrøm-Lang and Duspiva 1936; Powning, unpublished data; Powning, Day, and Irzykiewicz 1951; Waterhouse 1952*a*, 1952*b*). However, except for some observations of Pradhan (1949), the record that at least some species are unable to digest spongin (Arndt 1931), and the record of the presence of free — SH groups in the midgut (Duspiva 1936), there is no information available on the physiology of digestion of dermestid larvae. These larvae, together with Mallophaga (chewing lice) and the larvae of a few species of moths, are the only animals thought or known to be capable of digesting keratin. The report (Stankovic, Arnovljevik, and Mataverlj 1929) that the crop juices of a hawk, but not of a vulture, were capable of digesting feather keratin requires confirmation.

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It is of great interest, therefore, to examine the mechanism of keratin digestion in dermestid larvae, and to determine whether it follows the pattern already established for *Tineola* larvae. A number of features of digestion in dermestid larvae are described herein and these enable a comparison with *Tineola* to be made, which indicates both similarities and important differences.

II. Methods

Almost all of the experiments were carried out with three species, *Anthrenocerus australis* (Hope), *Anthrenus verbasci* (L.), and *Attagenus piceus* (Oliv.), although *Anthrenus vorax* (Waterh.) and *Dermestes maculatus* Deg. were also used in some experiments. All cultures were maintained in "Agee" jars containing a layer about 1 in. deep of a diet of casein 83 per cent., finely powdered yeast 15 per cent., cholesterol 1 per cent., and Hubble's salt mixture 1 per cent. Folded pieces of woollen fabric (air-dried after dipping in a suspension of 5 g. finely powdered yeast and 0.25 g. cholesterol per 100 ml. water) were placed on top of the powdered diet. The gauze tops of the jars and the upper surface of the fabric were sprinkled lightly with the active ingredient of "Dimite,"* which prevented mite infestation without apparently affecting the insects in any way. Adults were kept under similar conditions but transferred at weekly intervals to fresh jars, producing larval cultures of approximately known age. Adults were also supplied with drinking water and 15 per cent. honey in water in cotton-wool-plugged tubes after it had been found that they lived better in the presence of free moisture. All stock cultures were maintained at 30°C. and 75 per cent. relative humidity.

In the dye and metal feeding experiments single larvae were kept at 30°C. and 94 per cent. R.H. (over a saturated solution of potassium nitrate) in individual gauze-covered tubes with a piece of treated fabric. The woollen fabric (either unreduced, or with disulphide bonds partially reduced by brief treatment with 0.5M sodium thioglycollate at 40°C. and pH 10) was first dipped in 70 per cent. alcohol to ensure wetting, washed in water, and then steeped in a solution of the desired compound in a filtrate of the yeast-cholesterol suspension mentioned previously. The treated fabric was air-dried before use. This procedure of keeping single larvae in tubes was necessitated by their rather irregular feeding habits. Thus, for some days before and after moulting and, indeed, at other times also, larvae might cease to feed. By examining the tubes daily for faeces it was possible to select larvae whose alimentary tract was filled with the food in question.

When highly coloured food was present in the beginning of the midgut this could be seen through the cuticle of the metathorax and first abdominal segment if the larva was examined ventrally under CO_2 anaesthesia. This proved a useful check on the colours of pH and redox indicators in the midgut of living larvae.

* 1,1-Bis(*p*-chlorophenyl)ethanol, Sherwin Williams Co., U.S.A.

III. Results

With relatively few exceptions, which are specifically mentioned, *australis, verbasci,* and *piceus* larvae gave similar results in the experiments listed below.

(*a*) *Morphology of the Alimentary Canal*

Several authors (Braun 1912; Lison 1937; Mobusz 1897; Pradhan 1949) have dealt with various aspects of the morphology and histology of the alimentary canal of dermestid larvae. The foregut and midgut (Fig. 1 (*a*) and (*b*)) are simple, uncoiled, and possess no crypts or diverticula. The hindgut first runs forwards and then turns back upon itself to lead to the anus. There are six malpighian tubules which, after running to various regions of the abdomen, become associated in two pairs of three. These two groups then fuse and disappear, where the hindgut bends back on itself, into a sheath investing one side of the hindgut, and thus produce the cryptonephridial arrangement characteristic of the larval malpighian tubules of many Coleoptera, Lepidoptera, and ant lions (Lison 1937; Wigglesworth 1951). This arrangement is thought to play an important part in the conservation of water and salts. The anterior end of the hindgut in dermestid larvae is attached to the cryptonephridial sheath where it becomes enlarged in the region of the rectum.

Mobusz (1897) considered that a peritrophic membrane was absent in *Anthrenus verbasci,* although Aubertot (1934) recorded for *Attagenus pellio* the presence of a membrane that became more distinct and multiple-layered as it passed down the gut. It is true there is no well-defined membrane arising at the level of the oesophageal invagination. However, in the species examined in the present study (and particularly in *piceus*—Plate 1, Fig. 2) a membrane enclosing the food is clearly visible in the posterior half or two-thirds of the midgut. In *piceus* the membrane can sometimes be traced almost to the anterior end of the midgut, in *verbasci* it is often difficult to trace it anteriorly beyond the middle of the midgut, whereas *australis* occupies a position intermediate between these two species. Where it is first visible the membrane is rather diffuse in nature and its origin is uncertain. From the middle or posterior third of the midgut onwards, however, it becomes relatively tough and well defined and can be removed without damage with enclosed food. Its mechanical properties under these conditions indicate that it is an organized structure and not merely epithelial debris loosely enveloping the food.

(*b*) *Histology and Tracheation of the Midgut*

The histology of the midgut presents no unusual features. As observed by Mobusz (1897) there are small groups of regenerative cells, each separated by a variable number (5-20) of simple epithelial cells (Plate 1, Figs. 4-6). The latter have a conspicuous striated border, a small centrally placed nucleus, and a fine uniform cytoplasm with few or no granules visible with ordinary staining techniques. When granules were present they occurred in the cytoplasm between the nucleus and the gut lumen, but were not restricted to any particular region of the midgut. When tested by the Gallamine blue method

for calcium (Stock 1949) and by the rhodizonate method for barium and strontium (Waterhouse 1951) positive staining of the granules resulted. The granules also gave a weakly positive test for phosphate (cobalt sulphide method). Koehler (1920) reported the presence of numerous granules of calcium carbonate and oxalate in the midgut epithelium of *Dermestes fulvescens,* although it is not clear whether larvae or adults were examined. If

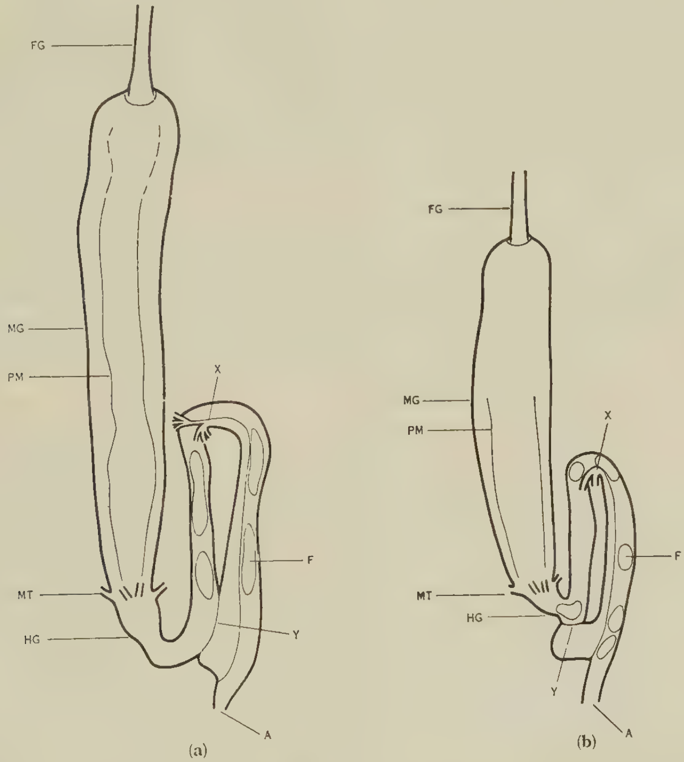


Fig. 1.—Ventral aspect of alimentary canal of dermestid larvae with hindgut rotated slightly to show entrance of malpighian tubules. (*a*) *Attagenus piceus.* (*b*) *Anthrenus verbasci. A,* anus; *F,* food mass in hindgut; *FG,* foregut; *HG,* hindgut; *MG,* midgut; *MT,* entrance of malpighian tubules into gut; *PM,* peritrophic membrane; *X,* entrance of malpighian tubules into cryptonephridial sheath; *Y,* region of attachment of anterior hindgut to cryptonephridial sheath.

larvae were used, either this species differs from those examined in the present study or the granules may be due to the diet (unspecified) upon which her insects were reared. It is of interest also to record that Braun (1912) mentioned a narrow zone of granules near the lumen border of the midgut epithelium of *Dermestes lardarius* larvae. The entire midgut epithelium appears to have a uniform structure and Bodian staining does not reveal any polymorphism of cell type as in *Tineola* larvae (Waterhouse 1952*a*). The entire epithelium is renewed at each moult (Braun 1912; Mobusz 1897).

It has already been noted that the midgut tracheation of *Anthrenus* and *Attagenus* larvae is less well developed than that of *Tineola* larvae which, in turn, has poor tracheation in this region compared with that of some other Lepidoptera (Day 1951*b*). Examination under dark field demonstrates that the midguts of last-instar *verbasci* and *australis* are supplied with very few tracheae and, occasionally, apparently with none at all. When tracheae are present they generally occur at the extreme anterior and posterior ends of the midgut, they branch very little, and they reach only a small area of midgut epithelium. The *vorax* midgut is better tracheated than either of these two species, although even it is supplied with only a few tracheae. Very occasionally in *verbasci* tracheoles are coiled upon themselves in an unusual fashion (Plate 1, Fig. 3) and the same tendency, although less marked, was also observed in *vorax, australis,* and *piceus.*

The tracheation of the *piceus* midgut is quite different, since there are many tracheae, which supply air to a good deal of the surface of the midgut (Plate 1, Figs. 1 and 2). Even this tracheal supply cannot, however, be regarded as particularly rich, since it is not unlike that of *Tineola* larvae. The *maculatus* midgut is more richly tracheated than any of the other species examined and it would appear to be more nearly comparable in this respect to the midgut of many other insects.

(*c*) *Microscopical Examination of Wool undergoing Digestion*

Pieces of wool in the digestive tract vary in length from about 40 to 120 μ, the average size increasing somewhat with advancing larval development.

When the digestive tract is examined under polarized light the wool in the anterior portion of the midgut is seen to be strongly birefringent. Further down the midgut many of the wool fragments have lost much of their birefringence, although others are little changed. The conspicuous and abrupt change in birefringence observed about one-third way down the midgut in *Tineola* larvae (Day 1951*a*) does not occur in dermestid larvae. This may be due, in part, to the fact that the wool is not "packeted" and firmly enclosed throughout the entire midgut in a well-defined peritrophic membrane as in *Tineola.* As a result, some mixing of wool fragments by peristaltic movements may be permitted.

In the posterior third of the midgut and in the hindgut partly detached scales can sometimes be seen and striations are conspicuous in many of the fibres, due no doubt to the removal of some intracellular cementing substance. Most, but not all of the faecal pellets contain wool fragments in various stages of digestion. Most of these fragments are either conspicuously striated or the original wool fibres have broken down into slender microfibres.

The presence of incompletely digested wool in the faeces is, perhaps, not surprising in rapidly feeding larvae, which may produce as many as 300 faecal pellets per day, although 30-100 is a more usual number. From counts of faecal pellets produced after removing feeding larvae from fabric and from feeding dyed fabrics, it appears that food may frequently pass completely through the digestive tract in 8-12 hr. at 30°C. (cf. 8 hr. for *Tineola* (Day 1951*a*)).

(*d*) *The pH of the Digestive Tract*

Table 1 shows the results obtained when larvae were fed on fabrics impregnated with saturated solutions of pH indicators. The contents of the entire midgut generally had a pH of 6.8-7.0 (green with brom-thymol blue). There was a certain amount of variation, however, since in a small proportion of larvae phenol red, and less frequently cresol red, exhibited a pinkish orange colour in the midgut. If the gut wall of these larvae was punctured and the contents spread out it could be seen that neither indicator was displaying its full alkaline coloration, which was readily produced on addition of dilute alkali. It appears therefore that at times the midgut pH may be as high as 8.2. On no occasion did thymol blue change colour, indicating a pH of less than 8.4. Pradhan (1949) records the pH of the midgut contents of *Anthrenus vorax* (= *fasciatus*) as 6.8.

TABLE 1

pH OF ALIMENTARY CANAL CONTENTS OF THE MAJORITY OF INDIVIDUALS OF THREE SPECIES OF DERMESTID LARVAE

Indicator	Midgut	Hindgut
Brom-phenol blue	>4·0	>4·0
Brom-cresol green	>4·6	4·4–4·8
Chlor-phenol red	>5·8	<5·8
Brom-cresol purple	>6·2	<6·0
Brom-thymol blue	6·8–7·0	<6·7
Phenol red	<7·8	<7·6
Cresol red	<7·8	<7·8
Thymol blue	<8·4	<8·4
Range	6·8–7·0	4·4–4·8

The pH of the hindgut and faeces was 4.4-4.8. However, in occasional larvae of *australis* the value ranged to lower than pH 2.0, some faeces from these larvae being red and some yellow after feeding on thymol blue.

The zone of pH change between midgut and hindgut varied a little, according to how recently before examination food had passed on. Normally the change occurred about the level of entry of the malpighian tubules, but occasionally it was slightly further down the hindgut, particularly in *australis*.

When larvae were fed on fabric treated with dilute indicator solutions there was little colour visible in the midgut, although the contents of the hindgut were often definitely coloured. This suggests that indicators may be absorbed by the midgut and either discharged into the hindgut via the malpighian tubules or the hindgut epithelium. However, in none of the larvae was there any visible accumulation of indicators in the gut epithelium or in the malpighian tubules.

(*e*) *The Oxidation-reduction Potential of the Digestive Tract*

The results obtained by feeding oxidation-reduction indicators with the diet are shown in Table 2. If the midgut pH is taken to be 7.0 (Table 1), the fully reduced colour of potassium indigo disulphonate and the partly oxidized colour of brilliant alizarine blue indicates a midgut potential in the range — 190 to — 200 mV. (Hewitt 1950).

TABLE 2

OXIDATION-REDUCTION POTENTIAL OF THE ALIMENTARY CANAL CONTENTS OF THREE SPECIES OF DERMESTID LARVAE

Indicator	E'_0 at pH 7 (mV.)	Condition of Indicator in	
		Midgut	Hindgut
1-Naphthol 2-sulphonate indophenol	+123	Fully reduced	Oxidized
Thionine	+ 63	Fully reduced	Oxidized
Methylene blue	+ 11	Mostly fully reduced	Oxidized
Indigo tetrasulphonate	— 46	Fully reduced	Oxidized
Tetrazolium blue		Fully reduced	Reduced
Indigo trisulphonate	— 81	Fully reduced	Oxidized
Indigo disulphonate	—125	Fully reduced	Oxidized
Brilliant alizarine blue	—173	Partly reduced	Oxidized
Phenosafranin	—252	Oxidized	Oxidized
Range		—190 to —252 mV.	+260 mV.

In individuals with a pH of about 8.0 (see earlier) a potential in the vicinity of — 230 mV. is indicated.

Methylene blue gave somewhat anomalous results, some of the fabric in the midgut exhibiting a pale bluish grey coloration. This, however, was very much paler than the deep bluish grey coloration of the ingested food. indicating a considerable degree of reduction. When the larvae were dissected under 0.1 per cent. triphenyltetrazolium chloride no pink or red colour was produced, this differing markedly from the results obtained for *Tineola* larvae in which reduction occurred (Day 1951*a*). The negative results in dermestid larvae may possibly be due to the absence of sufficient endogenous substrate in the gut for reduction to occur under these conditions.

Little is known of the systems responsible for the maintenance of reducing conditions in the midgut. It is highly probable that cysteine is present, since the contents give an intensely positive nitroprusside reaction for — SH groups. Furthermore, both Pradhan (1949) and Powning (unpublished data)

record the presence of cystine in the faeces of dermestid larvae. Ingested tetrazolium blue is reduced in the midgut but this reduction probably cannot be effected by cysteine at the pH (7.0) of the digestive juices (Rutenburg, Gofstein, and Seligman 1950), although G. Rogers (personal communication) obtained reduction of the related triphenyltetrazolium chloride in the presence of 0.08M cysteine at pH 7.4 and 35°C. Hydrogen sulphide reduces tetrazolium blue quantitatively, but evidence from metal metabolism (see below) suggests that little or no H_2S is formed during digestion in dermestid larvae. The production of the blue formazan may probably therefore be taken as evidence of dehydrogenase activity in the midgut (Rutenburg, Gofstein, and Seligman 1950).

In the hindgut a potential more oxidizing than + 260 mV. is indicated.

As a general rule the indicators were not accumulated in visible quantities by the epithelial cells. However, methylene blue is accumulated by some larvae in the epithelium of the anterior fifth of the midgut, and by others in the posterior half of the midgut, although in most larvae no accumulations were seen. Occasionally the cells of the malpighian tubules and fat body adjacent to the midgut contained oxidized methylene blue. Oxidized indigo disulphonate was accumulated by one *australis* larvae at the very posterior end of the midgut.

(*f*) *Metal Feeding Experiments*

Larvae were fed on woollen fabric impregnated with many of the salts used in the *Tineola* digestion experiments (Waterhouse 1951*a*). Faeces produced after ingesting the great majority of salts (e.g. those of Ni, Fe, Cd, Cu, Pb, Hg) were not, as a rule, any different in colour from those of control insects, even when the impregnation solution was concentrated (e.g. 15 per cent. $NiSO_4$. Where the fabric was coloured by the impregnation treatment (e.g. $HAuCl_4$), the faeces had the same colour as the fabric ingested. A small number (less than 0.5 per cent.) of larvae gave anomalous results in that dark faeces were produced when they were fed on lead or copper-impregnated fabrics and these larvae apparently produced sulphides. It is clear, however, that, with these possible exceptions, the larvae do not produce metal sulphides, which are so characteristic of the metal metabolism of *Tineola* larvae (Waterhouse 1951*a*). By transferring the larvae that had produced what are provisionally regarded as sulphides to thymol blue fabric it was shown that there was no correlation between capacity for sulphide formation and unusually low pH of the faeces (see earlier).

It appeared at first to be an anomalous result when larvae fed on fabric impregnated with 5 per cent. cobaltous chloride or saturated (3 or 4 per cent.) sodium tellurite or tellurate, had dark brown or black food respectively in the midgut and hindgut and produced brown or black faeces. However, on mixing equal volumes of M/10 cysteine and M/10 metal salts in either maleinate buffer at pH 6.9 (Smits 1947) or, if the buffer was incompatible, in water, cobalt produced a soluble dark brown reaction product and sodium tellurate slowly formed a finely divided black precipitate. Sodium tellurite produced initially a pale yellow precipitate that later blackened. Salts of other metals produced

white or nearly white precipitates (e.g. Zn, Cd, Sn, Pb, Hg, Ag) or lightly coloured or colourless solutions (e.g. Ni, Mn). Copper produced a light grey precipitate and iron a hydroxide-like precipitate, sometimes preceded by a transient blue colour. The implication is that metal-cysteine reactions may occur in the digestive tract of dermestid larvae, in contrast with sulphide formation, which is the predominant reaction in *Tineola.* Where the compounds are light in colour (all elements tested except cobalt and tellurium) the uric acid and other materials present in the faeces mask their colour and the faeces appear normal.

A search in the literature revealed that cobalt forms several complexes with cysteine, the cobaltic complex

$$(K_2Co^{+++}(OH)(\text{—}SCH_2CH(NH_2)COO^-)_2)_2$$

being the most stable, being soluble and having a dark, yellow-brown colour. The pH and oxidation-reduction potential in the dermestid midgut are such as to permit this stable complex to be formed. Its formation depends upon the presence in the complexing molecule of a —SH group and a —NH_2 group, although there is some doubt whether a —COOH group is also involved (Albert 1952). Thus thioglycollic acid will produce a brown cobalt complex, but not cysteine ethylated at the —SH group. Cystine alone gives no colour reaction with cobalt (Michaelis 1929; Michaelis and Barron 1929; Michaelis and Yamaguchi 1929; Michaelis and Schubert 1930; Schubert 1931). It is highly probable that the dark brown colour produced in the dermestid gut following ingestion of cobaltous chloride is a complex formed with the cysteine or cysteine-peptides produced by digestion of keratin, particularly since the material responsible for the colour in the faeces was water-soluble as is the cobalt-cysteine complex produced *in vitro* (Kendall and Holst 1931; Shinohara and Kilpatrick 1934).

The formation of a white crystalline mercury-cysteine complex from mercuric and mercurous salts and from metallic mercury is recorded (Barron, Flexner, and Michaelis 1929), as is also a red, weakly coloured (in dilute solution) nickel-cysteine complex (Michaelis and Barron 1929). Iron, copper, and manganese catalyse the oxidation of cysteine to cystine by the formation of complexes (Michaelis 1929; Michaelis and Barron 1929), but there is no indication that highly coloured stable complexes are ever formed with these metals. The results of feeding metals to dermestid larvae, therefore, are consistent with the hypothesis that metal-cysteine reactions occur in the midgut.

Little information is available on the nature of the black material formed by feeding sodium tellurite, although it may be metallic tellurium, since this substance is readily reduced to the elementary condition. After larvae had fed on the tellurium fabric for some time a small zone of cells at the anterior end of the midgut sometimes became dark with accumulated material. However, the amount accumulated was too small to permit histological localization in the cell. Where food was present in the foregut, it was black, and mixing of the contents of the foregut and midgut by peristaltic and anti-peristaltic action could sometimes be observed. When larvae were fed on ferric chloride fabric and tested by the Prussian blue reaction, ferric iron could be demonstrated throughout the midgut, there being no regular zones in which particularly large concentrations appeared.

(g) *Comparison of Cysteine Breakdown in Dermestid and* Tineola *Larvae*

From evidence presented above and by Waterhouse (1952*a*) it appears that hydrogen sulphide is produced at least transiently by *Tineola* larvae, but not by dermestid larvae, its source being the cysteine formed by digestion of keratin. A greater degree of degradation of cysteine in *Tineola* than in dermestids is supported by the analyses of Powning (unpublished data), who found that *piceus* faeces contain about 12 per cent. cystine, compared with 6-7 per cent. for *Tineola* larvae.

This difference in capacity for cysteine breakdown may be due to the possibilities that H_2S is liberated at pH 10 (in *Tineola*) but not rapidly enough (or not at all) at pH 7 (dermestids) to produce visible sulphide formation, or that *Tineola* larvae possess a desulphydrase capable of splitting off H_2S from cysteine (Fromageot 1951; Symthe 1945), but that this is absent or very weak in dermestid larvae. Irrespective of the presence of a desulphydrase it is highly probable, from what is known of the ease of cleavage of carbon-sulphur bonds (Tarbell and Harnish 1951) that cysteine and cysteine peptides would liberate H_2S very much more readily at pH 10 than at pH 7. However, there is some evidence that splitting of H_2S in *Tineola* larvae is, in part, under enzyme control. It is known that cysteine desulphydrase is completely inhibited by 0.001M (or less) hydroxylamine or semicarbazide (Lawrence and Symthe 1943). When *Tineola* larvae were fed on woollen fabric that had been soaked in a 10 per cent. solution of either inhibitor plus 5 per cent. nickel sulphate, some nickel sulphide was produced in the midgut and appeared in the goblet cells and in the faeces.

However, the amount of nickel sulphide produced was distinctly less, and the colour of the faeces far lighter, than in controls on nickel sulphate alone. Larvae fed on inhibitor plus lead acetate fabric showed a similar, but less marked inhibition of sulphide formation. When *Tineola* larvae were fed on silk that had been immersed in solutions containing 5 per cent. nickel sulphate and 1 per cent. cysteine hydrochloride, or 3 per cent. methionine or glutathione with and without 10 per cent. inhibitor, there was, once again, an indication of some inhibition of sulphide formation in the presence of inhibitors. In spite of the fact that larvae feeding on all inhibitor-treated foods produced fewer faeces than usual (and hence ingested less food and nickel sulphate) there were clear indications that sulphide formation was being interfered with. Furthermore, since these experiments were carried out, the presence of a desulphydrase has been demonstrated *in vitro* using extracts of *Tineola* larvae (Powning, unpublished data).

The results with silk and sulphur compounds suggest that there is more than one H_2S-splitting enzyme present, since cysteine desulphydrase is unable to split H_2S from methionine (Fromageot 1951). Alternatively the enzyme may be a non-specific desulphydrase unless the methionine and glutathione are first transformed to cysteine in the larval midgut before H_2S is split off, which is improbable.

Lower concentrations of inhibitor (5 or 1 per cent.) produced progressively less marked effects. This is possibly due to the fact that the inhibitors may

easily combine with other compounds in the larval gut and may not be available for desulphydrase inhibition. The continued production of small amounts of sulphide in the presence of inhibitor either indicates that inhibition is incomplete or that a significant amount of H_2S is liberated under the alkaline conditions (pH 10) by a purely chemical reaction without the intervention of an enzyme.

(*h*) *Digestion of Silk*

Raw silk thread consists of two homogeneous strands of water-insoluble silk fibroin (forming some 75 per cent. of the fibre) cemented together and surrounded by sericin. Sericin may be separated into three components, two (sericins *A* and *B*) being water-soluble and the third (sericin *C*) water-insoluble. Sulphur-containing amino acids have not been detected in silk, although there is a small amount of inorganic sulphide associated with sericin *C* (Shaw and Smith 1951).

Ever since the work of Abderhalden (1925) it has been believed that larvae of *Anthrenus museorum* are capable of digesting silk. However, there are several features about his experiments that throw doubt on the validity of this claim, at least so far as silk fibroin is concerned. Firstly, Abderhalden's analyses, which showed the sulphur content of his *museorum* larvae to be 0.5 per cent., suggest very strongly that the silkworm cocoons on which they were bred were contaminated by other materials. It is possible that they may even have contained dead pupae. Secondly, he was unable to detect microscopically any digestion when silk was incubated with larval digestive juices, although digestion "appeared" to occur if the silk was first ground as finely as possible. Thirdly, it seems highly probable, in view of their well-known sterol and vitamin requirements, that the silk on which the *Anthrenus* larvae thrived for several generations was contaminated by other materials, which would not only supply growth factors, but may also have provided the proteins etc. required for development. More reasonable are Abderhalden's statements that larvae would not develop on pure silk fibroin or on Canton silk.

When *australis* larvae were transferred to degummed silk (i.e. sericins *A* and *B* had been removed) they ingested it readily. Microscopical examination under ordinary and polarized light failed to reveal any indication that the fibres had been attacked by digestive enzymes. The faeces consisted almost entirely of undigested silk, which showed many signs of mechanical damage caused during ingestion, but their size and form, including indentations caused by incomplete severing of the fibre by the mandibles, appeared to be identical in the midgut and in the faeces. Similar results were obtained with larvae feeding on clean, empty silkworm cocoons on which they lived many months without increasing in size and produced large numbers of faeces consisting mainly of undigested silk. In view of the high content of tyrosine in fibroin (some 10 per cent.) it seemed possible that, if digestion of silk occurred, any tyrosine in excess over metabolic needs would appear in the faeces, just as cystine is present after feeding on keratin (Powning, unpublished data). However, a Millon's test on a 0.1N HNO_3 extract of faeces from silk-fed larvae indicated that tyrosine was absent. There is thus no evidence yet available

to indicate that silk fibroin is digested, whereas there is good evidence that certainly no more than a minor amount of digestion takes place.

The position with regard to sericins *A* and *B* is rather different. Because of their water solubility they would be expected to be removed from the raw silk fibre during passage through the alimentary tract. It remains, however, to be shown whether the water-soluble sericins are actually degraded by the digestive enzymes although their amino acid composition (Shaw and Smith 1951) suggests that this is probable. On the other hand, Duspiva (1950) found that, although the sericinase-containing fluid regurgitated by the silkworm moth just before emergence dissolved the sericin and softened the silk, very little breakdown of sericin could be detected.

The position may be summarized, therefore, by saying that, whereas dermestid larvae may be able to obtain some nourishment from the water-soluble constituents of raw silk, they do not appear to be able to digest processed silk (sericin *C* + fibroin), which forms the great bulk of the raw silk fibre. Available evidence indicates that this statement applies equally well to larvae of the clothes moth, *T. bisselliella.*

IV. Discussion

It is clear from these experiments that some species of dermestid larvae are capable of digesting wool and that, although the basic mechanism of digestion (reduction followed by enzyme attack) is similar to that in *Tineola* larvae, there are some notable differences in digestive physiology.

The histology of the midgut is simple, without differentiation into zones as in *Tineola.* Tracheation of the midgut is virtually absent in *australis* and *verbasci,* but in *piceus* is comparable with that of *Tineola,* which has relatively poor midgut tracheation (Day 1951*b*). This is not surprising since it would be difficult to conceive how intensely reducing conditions could be maintained in a midgut which is well oxygenated by means of a plentiful tracheal supply.

Some notable differences are evident when the present results for the pH of midgut contents are compared with those for *Tineola* larvae (Waterhouse 1952*b*). Firstly, no regions of varying pH were detected; secondly, there was no visible accumulation of indicator in the midgut epithelium of dermestid larvae; and thirdly, the midgut pH was very much less alkaline, falling within the range 6.8 to 8.2, compared with 9.8 to 10.0 for the most alkaline region of the midgut of *Tineola.* It appears that midgut pH may be more characteristic of the taxonomic group (although not necessarily of the order) to which a species belongs than of any unusual food habits possessed by individual species (Waterhouse 1949).

On the other hand, the midgut potential of — 190 to — 230 mV. is not very different from — 250 to — 280 mV. recorded for *Tineola* (Waterhouse 1952*b*). There is no reason to doubt that wool is effectively reduced at pH 7 and — 200 mV. in the dermestid midgut and it is possible that the materials responsible for the reducing conditions may also activate the proteolytic enzymes present. Lennox (1952), for example, has found that hydrosulphite

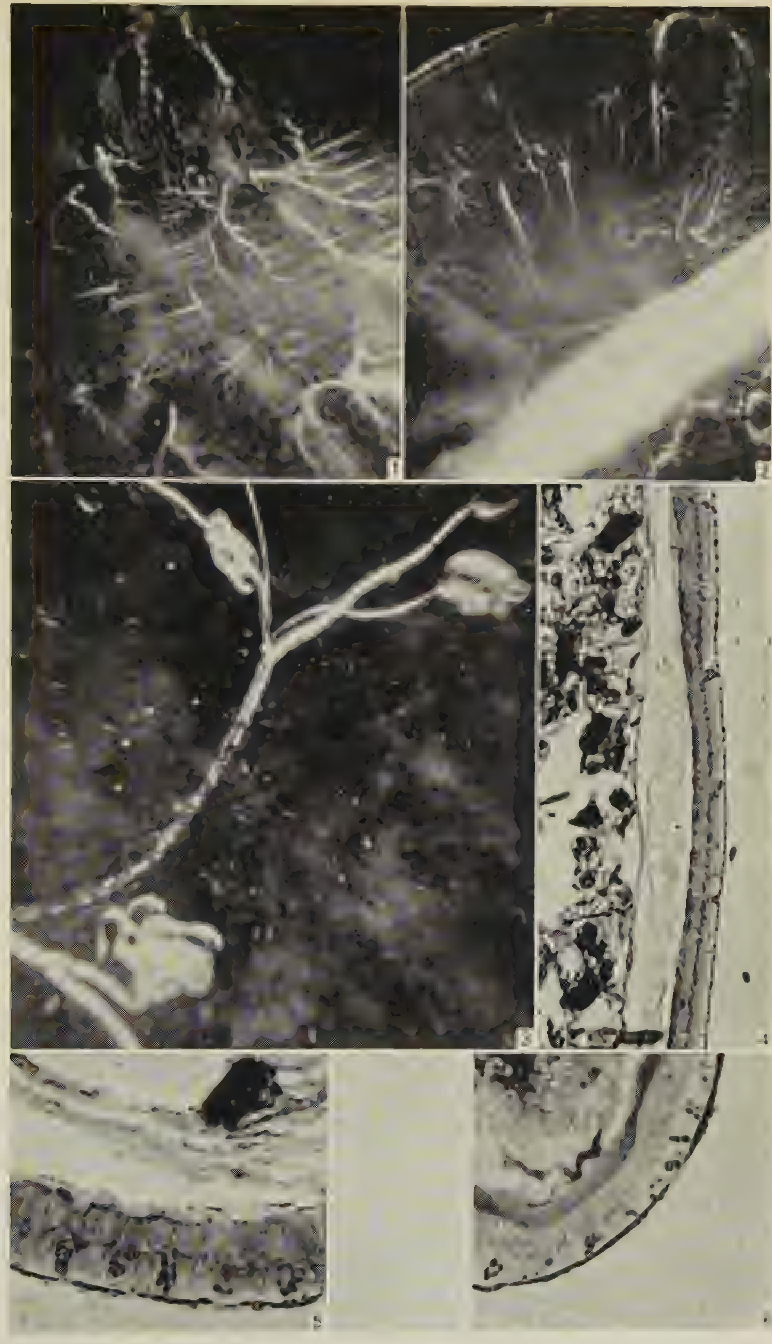
is less effective than bisulphite in promoting the digestion of wool by papain-urea in neutral solution although it is the stronger reducing agent.

It appears therefore that, in dermestid larvae, the disulphide bonds of wool cystine are rapidly reduced under the neutral reducing conditions encountered and that cysteine or cysteine peptides are formed as a result of the simultaneous action of proteolytic enzymes. The occurrence of this process is supported by the demonstration of an intensely positive nitroprusside reaction in the wool undergoing digestion.

There is good evidence that the breakdown of cysteine does not proceed as far in dermestid larvae as in *Tineola* larvae. In *Tineola* larvae, H_2S is produced in the midgut (partly chemically and partly enzymically) as can be shown by the formation of characteristically coloured sulphides from metals ingested with their food (Waterhouse 1952*a*). In view of the greater insolubility of most metal sulphides than their cysteine complexes, it is unlikely that appreciable quantities of metal-cysteine compounds are formed in the *Tineola* midgut in spite of the fact that cysteine is freely available, as evidenced by the presence of some 6-7 per cent. cystine in *Tineola* faeces (Powning, unpublished data). In the majority of dermestid larvae no sulphides are produced. Coloured faeces are only produced by elements (Co or Te) that were found to form deeply coloured reaction products with dilute solutions of cysteine. The salts of most metals do not produce strongly coloured metal-cysteine compounds and these resulted in the production of normally coloured faeces. Furthermore, although the figures cannot be compared directly, the fact that there is twice as much cystine in *piceus* faeces as in *Tineola* faeces (Powning, unpublished data) lends support to the belief that, in dermestid larvae, cysteine is not utilized to the same extent as in *Tineola* larvae.

The small proportion of dermestid larvae (both *australis* and *piceus*) that produce faeces apparently containing metal sulphides poses some interesting problems. Insufficient larvae have been detected so far to determine whether their midgut pH is higher than usual (i.e. whether this group is also responsible for the few pH records of about 8.0) and hence approaches more closely that of *Tineola* larvae. The differences in cysteine breakdown by *Tineola* and dermestid larvae and possible sulphide formation in occasional dermestid larvae are under investigation.

It is apparent from the absence of mortality after feeding on fabrics containing lead, mercury, etc. that dermestid larvae are capable of detoxifying many metals, although this problem has not been investigated specifically. Where detoxification occurs the process is somewhat analogous to that of *Tineola* larvae. Instead of insoluble sulphides being produced the metals form undissociated complexes with cysteine or cysteine peptides, which are either insoluble or, if soluble, exert less toxicity than the metal in ionized form. It is interesting that this detoxification appears to be no less effective than that in *Tineola* larvae, although fewer compounds have been investigated. However, it is possible that the long period of starvation dermestid larvae are able to survive may account for the low mortality produced by some of the treated fabrics.



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Explanation of Plate 1

Fig. 1.—An area typical of the most richly tracheated portion of the midgut of *A. piceus.* Dark field.

Fig. 2.—Typical tracheation of *A. piceus* midgut of starved larva, showing portion of contracted peritrophic membrane. Dark field.

Fig. 3.—One of the occasional tracheae supplying the midgut of *An. verbasci* showing coiling of tracheoles. Dark field.

Fig. 4.—L.S. midgut of *An. australis* larva showing simple epithelium, regenerative nidi, peritrophic membrane, and food in lumen. Mallory.

Fig. 5.—T.S. midgut of *An. australis* larva showing epithelium and peritrophic membrane in more detail. Mallory.

Fig. 6.—T.S. midgut of *An. verbasci* larva showing nuclei of epithelium, striated border, and fragment of peritrophic membrane. Bodian.

PROPERTIES OF THE MEMBRANES OF RAT AND RABBIT EGGS

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Summary

The properties of the membranes of rat and rabbit eggs—the zona pellucida, cumulus oophorus, and in the rabbit, the so-called "albumen" layer—have been studied. By histochemical techniques, it has been shown that all the membranes contain a large proportion of polysaccharide. The zonas are shown to be neutral or weakly acidic mucoproteins, being less acidic than the matrix of the cumulus oophorus, which is composed of hyaluronic acid and protein. Protein is demonstrable histochemically only in the zonas, but ready dissolution of the "albumen" layer and the matrix of the cumulus by pure proteinases indicates that protein forms an integral part of the structure of these layers. The term "albumen" is inappropriate, as the layer so designated has the properties of a strongly acidic mucoprotein.

The effects of hydrogen ion concentration and of oxidizing and reducing agents on the integrity of the membranes were investigated, and the results are discussed in relation to theories of sperm penetration of the zona pellucida. Although the rat zona is readily disintegrated by buffers more acid than pH 5, the rabbit zona requires buffers of pH 3 or lower. Of the oxidizing and reducing agents, hydrogen peroxide, with or without ascorbic acid, is the most effective.

The initial stages in the formation of the "albumen" layer have been detected by histochemical methods in eggs recovered from inseminated rabbits as early as 6-8 hr. after ovulation. This is discussed with reference to the suggestion that deposition of this layer provides an explanation of the short fertilizable life of the eggs in the rabbit. The inability of hyaluronidase to disperse the corona radiata of the tubal egg of the rabbit is probably due to the persistence of processes that interconnect these cells and also pass from the cells to the zona pellucida.

I. Introduction

Of prime importance in the study of fertilization of the mammalian egg is an understanding of the membranes surrounding it — the cumulus oophorus, the zona pellucida, and, in the rabbit, the so-called "albumen" layer.

The cumulus is a cellular layer with a rich intercellular matrix of a mucinous material. Early work on the disperson of the cumulus by proteolytic enzymes (Yamane 1930; Pincus and Enzmann 1936) indicated the presence in the matrix of peptide linkages. However, later investigators, who used streptococcal and testicular hyaluronidase, have shown that it is composed largely of the acid mucopolysaccharide known as hyaluronic acid (McClean and Rowlands 1942; Fekete and Duran-Reynals 1943). This material has also

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been demonstrated as a constituent of several different tissues (e.g. the subepithelial intercellular substance, synovial fluid, and umbilical cord) and its properties are now reasonably well understood.

On the nature of the zona pellucida there is less certainty. It is generally agreed that the zona contains polysaccharide, but little is known concerning its more specific classification. The polysaccharide was demonstrated by means of the Bauer technique in the zona pellucida of several different species (Walraff and Beckert 1939; Wislocki, Bunting, and Dempsey 1947). Its presence has been confirmed by Harter (1948) and Leblond (1950) who used the more specific periodic acid-Schiff technique. Wislocki, Bunting, and Dempsey (1947) also showed that the zona of the sow egg exhibited metachromasia with toluidene blue, a reaction that demonstrates acid polysaccharides, though not completely specific for these substances.

The presence of protein as well as polysaccharide in the zona was implied by Harter (1948) in his use of the term glycoprotein, but he presented no supporting evidence. Leach (1947) who stated that the zona was a mucoprotein, based his conclusion on the results of staining with the "mucin" stain, Bismarck brown. However, the presence of protein does not appear to have been conclusively demonstrated, and the relative amounts of polysaccharide and protein, and the character of these moieties, are little understood.

The nature of the third membrane under consideration, the so-called "albumen" layer of the rabbit egg, is least well understood. This material is laid down around the zona by the tubal mucosa and may well be of importance in limiting the fertilizable life of the rabbit egg (Hammond 1934). There appear to be no published reports on the nature or properties of this membrane.

In the present paper are described the investigations made to obtain more specific information about the three egg membranes listed above. It is hoped that the results obtained may be of use in gaining an understanding of the mechanism by which the sperm penetrates the egg.

II. Materials and Methods

Ovulation was induced in immature rats by the technique of Rowlands (1944), and in rabbits by the method described by Parkes (1943). The rat eggs were recovered from the fallopian tubes by dissection (Rowlands 1942) and the rabbit eggs by flushing with normal saline. Eggs were recovered from both species less than 6 hr. after ovulation, except when rabbit eggs with a thick layer of "albumen" were required, in which case they were recovered 24-48 hr. after ovulation.

Ovaries and tubal eggs were fixed in 4 per cent. basic lead acetate in 10 per cent. formalin or in 10 per cent. formol alcohol. Minchin's albumen method (see Gatenby and Beams 1950, p. 748) was used for embedding and sectioning tubal ova. Toluidene blue and thionin staining was carried out with a 0.1 per cent. dye concentration in 1 per cent. alcohol, and the sections were differentiated for 2 min. in 96 per cent. alcohol. The Bismarck brown and periodic acid-Schiff (P.A.S.) methods employed were those described by Leach (1947) and Hotchkiss (1948) respectively.

The xanthoproteic, Millon, and diazo methods, as described by Serra (1946), and the biuret reaction were used to demonstrate protein in whole, unembedded eggs fixed in a mercuric chloride-formalin mixture. The arginine reaction (after Baker 1947) was employed on sections of eggs.

The trypsin, chymotrypsin, and pepsin used were pure crystalline preparations manufactured by Armour Laboratories. The mould protease was a crystalline enzyme preparation from *Aspergillus oryzae* (Crewther and Lennox 1950).* The enzymes were used at a concentration of 0.05 per cent. in 0.1M phosphate buffers. The receptor-destroying enzyme and *Vibrio cholerae* mucinase† (Burnet 1948) were used as solutions in acetate buffers at pH 7.

Before investigating the effect of enzymes on the unfixed zona pellucida, the eggs were freed from the adherent cumulus by treatment with hyaluronidase, and placed in welled slides with the solution to be tested. The fluid was covered with paraffin to prevent evaporation and the preparation was incubated. The corona radiata of rabbit eggs was not removed by hyaluronidase but it could be taken off by flushing quickly with a fine pipette. The tendency for the eggs to stick to the internal surface of the pipette was overcome by rinsing the pipette with a very weak (approx. 1 : 10,000) solution of the wetting agent "Teepol" before use.

The method of Dempsey *et al.* (1947) was used to assess the strength of the acid dissociation of the polysaccharides in the egg membranes. Sections were stained for 20 hr. at 30°C. with 5×10^{-4}M methylene blue made up in 0.01M citric acid plus phosphate buffers.

III. Results

(*a*) *Histochemical Findings*

(i) *Tubal Eggs*.—When treated with toluidene blue, thionin, and safranin the matrix of the cumulus of rat and rabbit eggs and the "albumen" layer of rabbit eggs exhibited strong metachromatic reactions, which were not removed by washing with alcohol. The rabbit zona, however, showed only a weak reaction, which was readily removed by treatment with alcohol. The zona pellucida of the rat egg did not stain at all with toluidene blue and thionin but gave a weak orthochromatic reaction when stained with safranin. Both zonas stained lightly with the "mucin" stains, mucicarmine, and Bismarck brown, whereas the "albumen" layer and the cumulus stained heavily. All the membranes were strongly positive by the P.A.S. technique, which indicates that they contain polysaccharide. That this reaction was not due to lipid was shown by the absence of staining with Sudan black. The matrix of the cumulus showed an intense stain when treated by Hale's method, whereas the other layers showed little or none. The results obtained with eggs fixed in formol-

* Samples of the enzymes were kindly donated by Dr. F. G. Lennox, Wool Textile Research Laboratories, C.S.I.R.O., Melbourne.

† Samples kindly donated by Sir Macfarlane Burnet, Walter and Eliza Hall Institute, Melbourne.

alcohol were similar to those given by eggs fixed in formol-lead acetate. The tests for the presence of protein or amino acids in the matrix of the cumulus and the "albumen" layer were negative. The rat zona was stained by the diazo method but gave only a slight biuret reaction and no reaction to the arginine, xanthoproteic, and Millon tests. On the other hand, the rabbit zona was stained by all these methods. These results are summarized in Table 1.

TABLE 1

DEGREE OF STAINING OF THE MEMBRANES OF RAT AND RABBIT EGGS BY VARIOUS TECHNIQUES

Method Used	Rat Egg Zona Pellucida	Rabbit Egg Zona Pellucida	Rabbit Egg "Albumen" Layer	Matrix of Cumulus Oophorus, Rat and Rabbit
Toluidene blue: Before alcohol treatment	–	M+	M+++	M+++
Toluidene blue: After alcohol treatment	–	–	M+++	M+++
Thionin	–	–	M+++	M+++
Safranin	+	+	M++	M++
Mucicarmine	+	+	+++	+++
Bismarck brown	+	++	+++	+++
P.A.S.	+++	+++	+++	+++
Hale's method	∓	∓	+	+++
Sudan black	–	–	–	–
Arginine reaction	–	+++	–	–
Xanthoprotein reaction	–	++	–	–
Biuret reaction	∓	++	–	–
Diazo reaction	++	++	∓	–
Millon reaction	–	++	–	–

M = metachromasia.

Degree of staining indicated by — to +++ = none to intense.

The zonas of both species were homogeneous in appearance after staining but the "albumen" layer seemed to have a fibrous structure. No difference was apparent between the zonas of fertilized and freshly ovulated unfertilized eggs. Incubation of the sections with streptococcal or testicular hyaluronidase prevented the appearance of the metachromasia in the matrix of the cumulus only. The matrix cementing the cumulus cells and that between the coronal cells did not differ in this respect, nor in their histochemical reactions. An early stage in the deposition of the "albumen" layer around the rabbit egg was seen as a thin, strongly metachromatic band closely applied to the zona pellucida. This metachromasia was not affected by prior incubation of the sections with hyaluronidase. Traces of this metachromatic layer were seen in denuded eggs recovered from inseminated rabbits as early as 6 hr. after ovulation, and by 8-9 hr. after ovulation the layer was well defined. The albumen layer was not seen around eggs recovered from the tubes of uninseminated rabbits until 10 hr. after ovulation. By this time most of the eggs were free from the cumulus and the coronal cells.

The strength of the acid dissociation of the polysaccharide complexes present in the egg membranes was studied by the technique introduced by Dempsey *et al.* (1947) (Table 2). The rat and rabbit zonas were similar in their reactions, but the matrix of the cumulus had a slightly greater, and the "albumen" layer a much greater, dye-binding capacity at pH 4. The results obtained with smears of a hyaluronic acid preparation were very similar to those given by the matrix of the cumulus. Smears made from solutions of chondroitin sulphate gave similar results to that of the "albumen" layer.

TABLE 2

INTENSITY OF STAINING OF RAT AND RABBIT EGG MEMBRANES WITH METHYLENE BLUE SOLUTIONS BUFFERED AT VARIOUS pH

pH	Rat Egg Zona Pellucida	Rabbit Egg		Matrix of Cumulus Oophorus, Rat and Rabbit	Hyaluronic Acid	Chondroitin Sulphate
		Zona Pellucida	"Albumen" Layer			
2·0	–	–	+	–	–	+
3·0	–	–	++	–	–	++
4·0	∓	∓	+++	++	+	+++
6·0	+++	+++	++++	+++	++	++++
7·0	++++	++++	++++	++++	+++	++++

Degree of staining indicated by — to ++++ = none to intense.

(ii) *Ovaries*.—Wislocki, Bunting, and Dempsey (1947) noted that the intensity of the metachromatic reaction of the follicular fluid decreased as the size of the Graafian follicles increased. This was confirmed in the present study and a similar trend, though not so distinct, was seen in the intensity of the Hale, P.A.S., and "mucin" stains. Treatment with hyaluronidase prevented the metachromatic reaction but not the Hale or P.A.S. staining of the follicular fluid. The follicular fluid of the mature follicle reacted very weakly with toluidene blue but the matrix of the cumulus of the tubal egg, which is derived from the follicular fluid, showed an intense metachromatic stain. The reason for this difference in the degree of metachromasia of the hyaluronic acid gel before and after ovulation probably lay in the method used. All the tubal eggs used in this study were recovered from the fallopian tubes by dissection under saline. If the tube containing the eggs was fixed without dissection under saline the matrix of the cumulus exhibited only weak metachromasia. When a ripe Graafian follicle was punctured under liquid paraffin the liquor folliculi that emerged was quite fluid, but on contact with saline it became very viscous. The saline evidently induced contraction or polymerization of the polysaccharide mass. The former is the more likely and this suggests that the decrease in intensity of the metachromatic staining with increasing follicle size was due to an increasing dilution of the polysaccharide in the follicular fluid.

(b) *The Effects of Enzymes*

Fresh, unfixed eggs were used for all the tests. The zona pellucida of the rat egg was readily removed by trypsin, chymotrypsin, and a crystalline protease preparation from *Aspergillus oryzae* (Crewther and Lennox 1950), whereas the rabbit zona was only removed by trypsin (Table 3). Pepsin could not be used on the two zonas as the hydrogen ion concentration of the buffer was sufficient in itself to cause their dissolution. The "albumen" layer was disintegrated by trypsin, chymotrypsin, and pepsin but not by the mould protease, whereas the matrix of the cumulus was dispersed by all four enzymes. Pretreatment of the membranes with thioglycollate had no effect on the rate of digestion with trypsin, thus indicating the absence of keratin-like structures (Goddard and Michaelis 1934). The mucopolysaccharidases available, hyaluronidase, *Vibrio cholerae* mucinase, the receptor-destroying enzyme, and lysozyme, had no effect on the egg membranes with the exception of the depolymerization of the matrix of the cumulus by hyaluronidase.

TABLE 3

TIME TAKEN BY VARIOUS PROTEASES TO DISINTEGRATE FRESH RAT AND RABBIT EGG MEMBRANES

Enzyme concentration 0.05 per cent., temperature 37°C.

Enzyme	pH of Buffer	Rat Egg Zona Pellucida	Rabbit Egg		Matrix of Cumulus, Rat and Rabbit
			Zona Pellucida	"Albumen" Layer	
Trypsin	8	Removed in ½ hr.	Removed in 1 hr.	Removed in 2 hr.	Removed in 10 min.
Chymotrypsin	7	Removed in ½ hr.	Not removed	Removed in 2 hr.	Removed in 10 min.
Pepsin	2	*	*	Removed in 2 hr.	Removed in 20 min.
Mould protease	7	Removed in ½ hr.	Not removed	Not removed	Removed in 10 min.

* No test was possible as pH 2.0 buffer removed zona.

(c) *Effect of Hydrogen Ion Concentration*

The rabbit and rat zonas were readily disintegrated by acid solutions whereas the "albumen" layer of the rabbit egg was only dissolved by alkaline solutions (Table 4). The rat zona pellucida was removed by buffers below pH 5.5, as found by Hall (1935), but was not removed by buffers more alkaline than pH 5.5, though 0.1N sodium hydroxide caused some disintegration. The zona pellucida of the rabbit egg was removed by buffers having a pH of 3 or less and also by 0.1N sodium hydroxide. On the other hand, the dissolution of the "albumen" layer was not effected by solutions as acid as 0.1N hydrochloric acid, but was brought about by solutions more alkaline than pH 9. It was partly removed at pH 9.

(d) *Effect of Reducing and Oxidizing Agents, Urea, and Heat*

Solutions of the reducing and oxidizing reagents were buffered to bring the hydrogen ion concentration between pH 5 and 8. Fresh, unfixed eggs

were left in these solutions for periods up to 18 hr. at 37°C. The zonas of rabbit and rat eggs were affected by most of the reducing agents tried (Table 5). The organic reducing agents, cysteine, glutathione, and ascorbic acid, produced only a partial dissolution of the rat zona, whereas the two inorganic agents used, potassium metabisulphite and sodium hydrosulphite, were more effective. All the reducing agents produced only a partial disappearance of the rabbit zona, whereas the "albumen" layer was very little affected. Of the oxidizing agents, hydrogen peroxide was outstanding; even in low concentration (0.1 vol.) it removed the three membranes. This effect was greatly enhanced by the addition of 0.002M ascorbic acid, but the ascorbic acid solution by itself had little effect. Dissolution of the rat zona was effected by 0.1M potassium dichromate solution, but not by 0.01M potassium periodate. Neither of these solutions removed the rabbit zona and "albumen" layer. The rat zona was the only membrane dissolved by 2 or 4 per cent. urea. Both zonas quickly disintegrated in hot water but the "albumen" layer and the matrix of the cumulus were not affected by boiling in water for a short time.

Table 4

EFFECT OF HYDROGEN ION CONCENTRATION ON THE MEMBRANES OF FRESH, UNFIXED RAT AND RABBIT EGGS, OBSERVED AFTER 1 HR. AT 37°C.

pH	Rat Egg Zona Pellucida	Rabbit Egg	
		Zona Pellucida	"Albumen" Layer
2·0	Removed	Removed	Not removed
3·0	Removed	Removed	Not removed
3·5	Removed	Not removed	Not removed
5·0	Removed	Not removed	Not removed
5·5	Not removed	Not removed	Not removed
9·0	Not removed	Not removed	Partly removed
10·0	Not removed	Not removed	Removed
11·0	Not removed	Not removed	Removed
13·0	Partly removed	Removed	Removed

IV. Discussion

The specificity of the methods employed in this investigation determines in large measure the validity of the conclusions drawn. A study of the specificity of the histochemical methods employed for the demonstration of polysaccharides will be the subject of a separate communication. The following conclusions were drawn:

(i) The metachromatic reaction is not absolutely specific for acid polysaccharides but it can, if used in conjunction with other tests, furnish information as to the type of polysaccharide present.

(ii) The more acidic the polysaccharide the more intense is the metachromasia displayed. Likewise, the intensity of the staining of these compounds by "mucin" stains varies with the acidity.

(iii) If suitable precautions are taken the P.A.S. method is probably specific for substances that have adjacent glycol groups. However, not all polysaccharides react by this method, because many have these groups substituted. This is so with many of the sulphated polysaccharides.

(iv) The method devised by Hale (1946) for the demonstration of acid mucopolysaccharides is not specific for these substances.

TABLE 5

EFFECT OF VARIOUS OXIDIZING AND REDUCING AGENTS AND OF UREA ON THE FRESH, UNFIXED MEMBRANES OF RABBIT AND RAT EGGS

Reagent	Concentration	pH	Rat Egg Zona Pellucida	Rabbit Egg Zona Pellucida	Rabbit Egg "Albumen" Layer
Cystein	0·01M	6·0	Partly removed	Partly removed	Not removed
Glutathione	0·01M	6·7	Partly removed	Partly removed	Not removed
Ascorbic acid	0·01M	6·5	Partly removed	Partly removed	Not removed
Potassium metabisulphite	0·1M	5·5	Removed	Partly removed	Not removed
Potassium metabisulphite	0·01M	6·7	Removed	Not removed	Not removed
Sodium hydrosulphite	0·1M	6·3	Removed	Partly removed	Not removed
Potassium dichromate	0·1M	5·7	Removed	Not removed	Not removed
Potassium periodate	0·01M	7·0	Not removed	Not removed	Not removed
Hydrogen peroxide	0·1 Vol.	7·0	Removed	Removed	Removed
Hydrogen peroxide plus ascorbid acid (1:1)	0·1 Vol. plus 0·002M	7·2	Rapidly removed	Rapidly removed	Rapidly removed
Urea	2%, 4%	7·0	Removed	Not removed	Not removed

The evidence obtained by histochemical methods and treatment with proteolytic enzymes in the present investigation shows that the zonas of both rat and rabbit eggs contain protein. This fact does not appear to have been established previously. The observation that the rat zona is disintegrated by trypsin, chymotrypsin, and mould protease, whereas the rabbit zona is removed only by trypsin, indicates that the type of protein is not the same in the two species. The two proteases, chymotrypsin and trypsin, hydrolyse different peptide bonds, chymotrypsin acting at linkages involving the carboxyl end of tyrosine and phenylalanine residues, whereas trypsin acts at linkages involving the carboxyl end of lysine or arginine residues (Bergmann and Fruton 1941). Further evidence of the difference between the types of proteins in the zonas of the two species is afforded by the demonstration that the rabbit zona reacts to all the histochemical tests for protein used, whereas the rat zona only reacts to the diazo test. That the protein forms an integral part of the structure of the zona is indicated by the fact that proteases and other protein-denaturing agents such as urea and heat cause rapid disintegration of the membrane. Susceptibility to heat indicates weak protein linkages, whereas solution in acids, alkalis, or urea indicates the presence of electrovalent bonds (Brown 1950).

In confirmation of earlier work (Walraff and Beckert 1939; Wislocki, Bunting, and Dempsey 1947; Harter 1948) it has been shown that the zonas contain a large proportion of polysaccharide. Judging by the staining with methylene blue in buffers with pH < 6, and the reaction with toluidene blue and the "mucin" stains, the polysaccharides present are neutral or very weakly acidic in character. The polysaccharide of the rabbit zona is probably a little more acidic than that of the rat, as it reacts weakly with toluidene blue. It is concluded, therefore, that the zonas of rat and rabbit eggs are polysaccharide protein complexes that would be classified as neutral or very weakly acidic mucoproteins according to Meyer's (1945) terminology.

It may be profitable at this juncture to consider whether the known properties of the zona pellucida provide any clue to the means by which sperms penetrate it. Because the rat zona was removed by buffers with pH values as high as 4.5 or 5 (Hall 1935; Harter 1948), Harter has suggested that the acid produced in the metabolic processes of the sperm enables it to make its way through the zona. This, however, would not be possible in the rabbit as solutions as acid as pH 3 are needed to remove the zona in this species. In both species the zona is disintegrated by hydrogen peroxide, and it might be suggested that this compound, which is produced by sperms (Tosic and Walton 1946), enables them to penetrate the zona. If such a mechanism were effective, the sperms should also be able to penetrate the "albumen" layer, as this too is removed by peroxide. However, the "albumen" layer is generally recognized to be a bar to penetration (Pincus 1930; Hammond 1934; Chang and Pincus 1951).

The dissolution of the zona, at least in the rat, may be effected not only by an acid reaction and by hydrogen peroxide but also by other oxidizing agents and certain reducing agents. It may be suggested that any of these factors could be involved in the mechanism that permits the sperm to traverse the zona. A theory based on such a hypothesis, however, faces the objection that penetration of the zona does not readily occur *in vitro*, and indeed, as Austin (1951) points out, it is probable that penetration *in vitro* has never been conclusively demonstrated. Following the suggestion by Leblond (1950) and Austin (1951) that a specific enzyme may be involved in penetration, the zona of both rat and rabbit eggs was treated with several mucinases but these were without observable effect.

There does not appear to be any basis for use of the term "albumen" to designate the material that accumulates around the rabbit zona during its passage down the tube, because it is evident from the histochemical results that the "albumen" contains little protein. There must be some, however, as the layer is disintegrated by trypsin, chymotrypsin, and pepsin, though not by mould protease, urea, or hot water. These reactions also indicate that the type of protein present is different from that in the other egg membranes. The strong metachromatic, "mucin," and P.A.S. staining shows that this layer is composed largely of acid polysaccharide. The reason for the anomalous reaction by the Hale method is obscure as the chemical basis of this reaction is unknown. The ready removal of the "albumen" layer by alkali and by ascorbic acid and hydrogen peroxide confirms the presence of mucopolysaccharides, as

substances of this type are generally precipitated by acids and dissolved by alkalis (Haurowitz 1950, p. 200) and are depolymerized by ascorbic acid and hydrogen peroxide (Duran-Reynals 1942). That the polysaccharide moiety has an acidity similar to that of chondroitin sulphate is indicated by the intensity of the staining with methylene blue in acid solutions. The histochemical reactions of the polysaccharide of the "albumen" layer differ from those of most of the acid polysaccharides that have been isolated (Braden, unpublished data). On the basis of the results presented it may be concluded that the "albumen" layer is composed of an acid mucoprotein.

As already mentioned, the "albumen" layer may well have a special significance in limiting the fertilizable life of the rabbit egg (Pincus 1930; Hammond 1934). Pincus reported the presence of a very thin layer of "albumen" on eggs recovered from mated rabbits about 5 hr. after ovulation. Hammond concluded from his observations that the length of the fertilizable life of the rabbit egg is about 6 hr., and believed that the deposition of the "albumen" was responsible for this restriction in time. On the other hand, Chang (1951) states that the "albumen" layer is not present until about 14 hr. after ovulation and he considers that it is not the reason for the short fertilizable life. The results reported in the present paper tend to support the findings of Pincus, although it was not possible unequivocally to demonstrate the "albumen" layer earlier than about 8 hr. after ovulation. The determination of the actual time for which the rabbit egg remains penetrable to sperm must depend upon an assessment of the time taken by the sperm to reach the site of fertilization and on this matter the available data are at present inadequate.

The conclusion of earlier workers that the matrix of the cumulus oophorus is composed mainly of hyaluronic acid has been confirmed in the present investigation. Although it is not possible to demonstrate protein in the matrix by histochemical methods, it is evidently present in small proportions as the membrane is readily disintegrated by proteases.

The present results indicate that there is no difference between the histochemical reactions of the matrix of the cumulus and that of the corona radiata, a layer of densely packed cells immediately surrounding the zona. Sperm suspensions *in vitro* quickly disperse the cells of the cumulus, but in the rabbit the corona remains around the egg. However, both layers are removed from the egg *in vivo* within a short period (Pincus 1930). This suggests that the filaments connecting the coronal cells to one another and to the zona in the ovarian egg (Fischer 1905) are still present in the freshly ovulated egg. Examination of eggs soon after ovulation with the phase-contrast microscope reveals many processes passing from the coronal cells and ending on the zona pellucida.

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OESTROGENIC EFFECTS OF SUBTERRANEAN CLOVER: STUDIES ON THE FEMALE GUINEA PIG

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Summary

Oestradiol dipropionate injection (0.5, 2.0, 8.0, and 32.0 μg.) and subterranean clover ingestion (30 g. per day) produced similar effects in female guinea pigs, namely, infertility and epithelial changes in the accessory reproductive organs.

The "degree" of infertility obtained was directly relatable to the level of oestrogen supplied, being permanent at the 32 μg. level. The higher the dose given the longer the delay in recommencing breeding after stoppage of injections. However, clover consumption caused immediate, though transitory, infertility, recovery occurring simultaneously with cessation of feeding.

Injected oestradiol interfered with ovulation, and follicular atresia and gradual lengthening of the oestrus phase were regularly observed, but ingested clover oestrogen did not impede ovulation or the cyclic occurrence of oestrus.

It is suggested that the effect of oestradiol on the ovary was mediated via the pituitary by impairment of gonadotrophin production. On the other hand, the dietary (clover) oestrogen may have acted directly on the environment of the reproductive tract to modify the activity of the germinal elements.

I. Introduction

The work presented in this paper developed in conjunction with investigations of a specific disease of sheep grazing subterranean clover pastures (*Trifolium subterraneum* L. var. Dwalganup) in Western Australia. The disease is characterized by three clinical manifestations in the ewe, namely, infertility, maternal dystocia, and uterine prolapse (Bennetts, Underwood, and Shier 1946). These conditions result from the presence in the clover of a substance or substances with oestrogenic activity (Curnow, Robinson, and Underwood 1948; East, Underwood, and Bennetts 1949; East 1950); and the effects on the ewe are permanent or very prolonged (Underwood and Shier 1951). Moreover, the ingestion of the oestrogenically potent clover has no measurable effect on the oestrus incidence or on the breeding and non-breeding seasons of the infertile sheep (Underwood and Shier 1952). The meagre and somewhat conflicting data on the mechanism of the infertility produced by clover consumption prompted the present studies with guinea pigs. These animals had already been shown to respond to clover feeding in a manner similar in some respects to sheep and seemed, therefore, ideally suited to detailed laboratory investigation. Two series of experiments were planned and are described below. The first deals with the effects of injection of a natural

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oestrogen on the reproductive organs, oestrous cycle, and fertility of female guinea pigs and the degree of permanecy of these effects. The second deals with the effects, similarly assessed, of ingestion of "potent" subterranean clover.

The extensive literature detailing the influence of oestrogen administration on mammals has been well reviewed by Burrows (1949). Mention must be made, however, of an experiment with mice carried out by Emmens (1950), since the methods and objectives are to some extent comparable with those described in this paper. This worker rendered female albino mice sterile with injections ranging from 0.5 to 2.5 μg. of oestradiol benzoate given in oil twice weekly for a month. Even the lowest dosage caused total sterility during the period of treatment and for 2 weeks thereafter, although recovery at all levels was eventually complete. A relationship between the level of oestrogen supplied and the time required for recovery was indicated by the fact that the mice receiving the highest hormone dose took from 41 to 125 days to recommence breeding, whilst those at the lowest level recovered in 20-28 days.

II. Experimental

(a) Injection of Oestrogen

(i) *Preparation and Grouping of Experimental Animals.*—Fifty female and five male adult guinea pigs were randomized into groups receiving intramuscular injections of oestradiol dipropionate in 0.1 ml. peanut oil at various levels, namely, 0.5, 2.0, 8.0, and 32.0 μg. three times per week. The groups were constituted in such a manner that all the males contributed about equally to any fertility differences between members, e.g. five groups of 10 females and one male each. The composition of each group and the doses given were as follows:

2 females— 0.1 ml. peanut oil
2 females— 0.5 μg. oestradiol
2 females— 2.0 μg. oestradiol
2 females— 8.0 μg. oestradiol
2 females—32.0 μg. oestradiol
1 male —untreated.

At the end of 9 months, or after the second parturition, injections were discontinued to all animals and five females (one from each dosage level) killed. The remainder were then re-mated and the experiment continued for a comparable period of time.

(ii) *The Diet.*—Every group was supplied with an identical diet of supplementary ration and fresh green lucerne *ad lib.* The supplement consisted of bran 45 per cent., rolled oats 35 per cent., linseed meal 10 per cent., meat meal 8 per cent., salt 1 per cent., lime 1 per cent., moistened with water. Such a ration had proved adequate for both growth and reproduction in the past and therefore no change was made in its composition. In addition, each group had easy access to water, while the fresh lucerne provided a natural and constant high-level intake of vitamin C recognized as an integral requirement for guinea pigs kept under artificial conditions.

(iii) *Materials Used.*—The oestradiol dipropionate employed was a commercial product "Ovocyclin P" marketed by Ciba Ltd., in 1-ml. ampoules containing 5 mg. of the hormone in sterile oil. Stock solutions of requisite concentration were prepared with peanut oil and used as needed.

(iv) *Injection Technique.*—All the females were injected intramuscularly into the thigh, separate syringes being used for each dosage level. In order to avoid the possibility of local inflammation, alternate legs were used and the animals handled as gently as possible.

A set sequence of injection was adopted for individual groups and members within each group, then changed at regular intervals to minimize the chance of a particular animal (or animals) receiving the injection in the same order throughout the experimental period.

(v) *Methods of Comparison Adopted.*—Comparison of animals was made on the bases of vaginal patency, histological examination, weight of the reproductive organs, and breeding performances.

The condition of the vagina was checked and recorded on every alternate day throughout the entire experimental period. When the vagina was patent smears were taken and stained by the method of Shorr (1941) to determine the extent of the oestrus phase. The system of recording used was: open +, closed —, opening $\pm$, closing $\mp$, and the smear was recorded according to the presence and proportion of cornified cells, nucleated epithelial cells, and leucocytes. Finally, the average time of patency and closure was calculated for each animal and each dosage level both during treatment and after discontinuance of injections.

For histological examination and to obtain the weight of the reproductive organs, the reproductive organs of every guinea pig were removed, after sacrifice, by gross dissection to Bouin's fluid. Next, the individual glands were separated out, weighed, sectioned at 4-6 μ, and stained with Harris's haematoxylin and eosin. Permanent mounts were made of ovaries, fallopian tubes, uterus, and vagina.

For breeding performance, the following data were entered both on individual and summary record sheets: length of gestation; litter interval; weight of mother at parturition and at weaning; number, weight, and sex of young at birth and at weaning; and number and sex of any young born dead. The length of gestation was calculated by reference to the breeding cards showing details of vaginal patency, conception being indicated by the last opening before the series of minus signs showing the intervention of pregnancy. This has proved a relatively concise method.

The young were sexed and weighed as soon after birth as practicable, usually within the first half hour of life, and their body weights noted thereafter at weekly intervals until weaning. The piglets were weaned when they weighed 150 g. Maternal weights were recorded within a reasonable time of parturition and then at the regular weekly weighing, although a note was made of the body weight on weaning the last member of the litter.

Management of the guinea pigs near term was an important consideration. Since heavy losses of young occur in runs containing a number of adult animals, the experimental females were removed to individual breeding cages when parturition was judged imminent. Here they remained, separated from the male until weaning, when they were restored immediately to their particular group and mate. Nevertheless, injections continued for the entire time spent in the breeding cages. Such measures slowed the course of the experiment but were considered necessary for the collection of data relating to the viability of the young produced.

All the information was finally summarized and analysed by appropriate methods.

(*b*) *Feeding of Subterranean Clover*

(i) *Preparation and Grouping of Experimental Animals.*—Twenty female and 20 male adult guinea pigs were randomized into two groups each of 10 pairs. Group I received a "normal" diet of lucerne and supplementary ration *ad lib.* and acted as controls, while the members of group II were supplied with 30 g. dehydrated subterranean clover plus 30 g. mash per day. The paired animals were housed in separate cages under identical conditions.

The males were placed with their respective partners only at night, when copulation generally occurs, in order to minimize any consumption of clover on their part.

Five females of each group were sacrificed after dropping the second litter, or after 6 months, and the remaining animals continued on a normal (non-clover) diet for an identical period.

(ii) *The Diet.*—It was previously reported (East, Underwood, and Bennetts 1949) that fresh green clover proved fatal to guinea pigs within 2 days, and that in an endeavour to minimize these fatalities the material was air-dried at 100-130°F. and stored in 4-gallon air-tight tins. A similar diet was again supplied in this experiment and was found to be equally satisfactory. In addition, a supplementary ration as described in the first experiment was provided.

Every female in group I was given 70 mg. ascorbic acid per week dissolved in the drinking water during the period of time when clover was included in the diet. The males of this group did not require such supplementation as they were provided with fresh lucerne during the day while removed from the breeding cages. All the guinea pigs, both male and female, had access to separate drinking bottles containing 120 ml. water, which were renewed twice daily.

(iii) *Materials Used.*—The particular Dwalganup subterranean clover used in this investigation was grown at Kojonup in the south-west of Western Australia and cut in late September just prior to wilting. The oestrogenic activity of the sample was measured by the Allen-Doisy method on mice and 100 g. found to have a potency equivalent to 14 μg. oestradiol.

Daily intake figures for each female calculated from the residue weights showed that the clover was well consumed, the animals in group II eating an

average of 24 g. per day (30 g. total) for 177 days. By calculation, therefore, the animals ingested a total of 595 μg. oestradiol equivalent over the experimental period. This estimate is only approximate at best, as some clover, though only a small amount, must have been eaten by the males when returned to the experimental cages.

(iv) *Methods of Comparison Adopted.*—The animals were compared on the bases of vaginal patency, histological examination, weight of the reproductive organs, and breeding performance, and the appropriate data were collected as described for the previous experiment.

As the breeding pairs were housed separately there was no need for special management at parturition and the male was removed only during the regular daily periods. Post-partum mating was thus the rule.

Table 1

EFFECT OF OESTROGEN ADMINISTRATION AND WITHDRAWAL ON THE SEX CYCLE OF FEMALE GUINEA PIGS

Oestrogen Administered (Two gestation periods or 9 months) (10 animals/dosage level)				Oestrogen Withdrawn (Two gestation periods or 9 months) (Five animals/dosage level)		
Dose of Oestradiol Dipropionate (μg.)	Mean Time Vagina Open (oestrus) (days)	Mean Time Vagina Closed (days)	Mean Total Length Cycle (days)	Mean Time Vagina Open (oestrus) (days)	Mean Time Vagina Closed (days)	Mean Total Length Cycle (days)
—	3·4	15·9	19·3	3·9	14·4	18·3
0·5	4·0	12·6	16·6	3·2	14·1	17·3
2·0	6·1	10·1	16·2	4·8	14·7	19·5
8·0	7·5	7·7	15·2	4·0	8·8	12·8
32·0	26·4	5·4	31·8	8·3	6·9	15·2

III. Results

(*a*) *Injection of Oestrogen*

(i) *Vaginal Patency.*—The effect of increasing oestrogen dosage was reflected by the gradually increasing span of the oestrus phase, i.e. the time for which the vagina remained patent. However, the *total* length of the sex cycle was unaltered, except in the animals receiving 32 μg. oestradiol. Such treatment extended the cycle to approximately twice its normal length, and for the greater part of this time, as judged by the nature of the vaginal smear, the guinea pigs were in constant oestrus.

After the cessation of treatment the aberrant cycles returned to a more normal condition, although the oestrus phase of animals that had received maximal dosage was still considerably prolonged. The total length of the cycle was the same in all cases (Table 1).

(ii) *Histological Examination and Weight of the Reproductive Organs.*—Histological examination indicated a high incidence of atresia in the ovaries of all guinea pigs receiving oestrogen treatment. This was particularly obvious and advanced in the organs of animals given 32 μg. oestradiol. Here too, there was a definite lack of new and developing follicles and corpora lutea, which suggested that ovulation had not occurred in this group. The picture presented by the ovaries of the guinea pigs after cessation of treatment was still one of degeneration, though not as severe as in the injected animals.

In the 8 μg. group, atresia was also prominent although there were a number of mature follicles present. However, a more normal pattern of activity was recognizable after the injections were stopped, as corpora lutea were demonstrated for the first time.

Table 2

EFFECT OF OESTROGEN TREATMENT ON THE MEAN OVARIAN WEIGHT OF FEMALE GUINEA PIGS

Oestrogen Administered			Oestrogen Withdrawn	
Dose of Oestradiol Dipropionate (μg.)	Animals	Mean Weight Ovaries (R+L) (mg.)	Animals	Mean Weight Ovaries (R+L) (mg.)
—	5	115·84	5	138·30
0·5	5	84·86	5	80·28
2·0	5	95·00	5	125·38
8·0	5	89·74	5	92·28
32·0	5	64·04	5	106·72

The glands of animals treated with 2 μg. oestradiol again showed marked atresia, though both developing and mature Graafian follicles were evident. No luteal tissue was present. Genesis, development, and maturation of follicles was the rule in the ovaries of members of this group recovering from previous oestrogen treatment, while luteal activity indicated a return to the normal functional state.

Although atretic symptoms were directly relatable to the lowered fertility of guinea pigs on the minimum dosage level (0.5 μg.), follicular development was quite marked and it was not surprising that the organs of animals killed at the end of the experiment were strictly comparable to those of the controls.

The accessory reproductive organs—fallopian tubes, uterus, and vagina—of all guinea pigs, whatever the level of injection, showed the characteristic sequelae of oestrogen treatment. Hyperplasia, metaplasia, and keratinization were all present, though in varying degrees, and gradients of responsiveness were similarly evident. For instance, the lining membrane of the uterus and vagina was affected more severely than the epithelium of the fallopian tubes, and there was a gradient from the uterine horn to the region of the cervix, the latter being the more sensitive. While all the sex organs presented a regression towards the normal histological picture after cessation of treatment, there

was still a residuum of effect apparent in these glands. It was noticeable also that a reverse gradient of recovery existed, the fallopian epithelium recovering more quickly than the endometrium or the vaginal mucosa.

Only the ovary reacted to oestrogenic stimulation in a demonstrable manner, by showing a decrease in weight as compared with the control animals. There was, however, no significant difference between the ovarian weights of animals receiving different levels of oestradiol (Tables 2 and 3).

The uterine, vaginal, and oviduct weights did not give evidence of a definite organ reaction to the oestrogen supplied. This was rather surprising, but a high degree of variation in the organ weights may explain the finding. There was no indication of a significant variation due to the time of killing in any of the reproductive organs weighed, i.e. the discontinuance of oestrogen injections had had no effect on the weights of these glands when examined 9 months later.

Table 3

EFFECT OF OESTROGEN TREATMENT ON MEAN OVARIAN WEIGHT OF FEMALE GUINEA PIGS — ANALYSIS OF VARIANCE

Variance	Degrees of Freedom	Sum of Squares	Mean Squares	*F*	*P*
Male	4	12309·66	3077·42	3·35	<0·05
Killing	1	2641·20	2641·20	2·88	
Dose	4	1276·00	319·00	<1	
Error	40	36708·80	917·72	<1	
Total	49	52935·66			
Controls *v.* treated	1	8259·98	8259·98	9·00	<0·01
Residual	3	4049·68	1349·67	1·47	N.s.

(iii) *Breeding Performance.*—The results summarized in Table 4 show that oestrogen injection resulted in infertility as judged by lack of conception, and that the extent of the infertility depended on both the dosage level and the period of time for which the injections were given.

High-level administration of oestradiol dipropionate (8 and 32 μg.) was immediately effective in preventing conception, though a small proportion of the guinea pigs receiving 2 μg. of the hormone dropped one litter. Further treatment of these animals produced complete infertility. While total sterility was not induced by the minimum dose of 0.5 μg. oestrogen, the small number of animals conceiving for a second time suggested that here the actual injection period was of considerable importance. In this case, it was reasonable to assume that infertility would have resulted if the injections had been continued for a longer period.

Data pertaining to the permanency of these effects are tabulated in Table 5, the figures in column 5 being calculated by subtracting the mean time for the controls (107 days) from the rest. It was evident that, although permanent

infertility resulted from treatment with 32 μg. oestradiol for the experimental period adopted, the other animals recommenced breeding at intervals depending, to some extent, on the level of oestrogen received. While the delays occasioned by injection of 0.5 and 2.0 μg. were not very different, the effect of the 8.0 μg. dose was very marked. The indications were that the higher the dosage the greater the delay.

TABLE 4

FERTILITY OF FEMALE GUINEA PIGS AFTER OESTROGEN ADMINISTRATION AND WITHDRAWAL

Dose of Oestradiol Dipropionate (μg.)	Litter 1			Litter 2			Litter 1*S* (3)*			Litter 2*S* (4)*		
	Animals	Fertile	Fertility (%)	Animals	Fertile	Fertility (%)	Animals	Fertile	Fertility (%)	Animals	Fertile	Fertility (%)
—	10	10	100	10	10	100	5	5	100	5	5	100
0·5	10	6	60	10	2	20	5	5	100	5	5	100
2·0	10	3	30	10	0	0	5	4	80	5	4	80
8·0	10	0	0	10	0	0	5	4	80	5	2	40
32·0	10	0	0	10	0	0	5	0	0	5	0	0

* Litters 1S and 2S refer respectively to the first and second litters after discontinuance of injections. Injections were stopped after litter 2.

The breeding details of all fertile females used in this experiment were obtained. Since the fertile periods of the treated guinea pigs were not full, contemporaneous, or of the same duration, no significance could be attached to actual litter numbers but only to the figures relating to the survival and

TABLE 5

DELAY IN REGAINING FERTILITY CAUSED BY OESTROGEN TREATMENT

Dose of Oestradiol Dipropionate (μg.)	Litters	Mean Time to First Litter* (days)	Range (days)	Delay (days)
—	10	107·4	89—123	0
0·5	5	113·6	87—146	6
2·0	4	114·0	93—134	7
8·0	4	139·8	65—191	32
32·0	—	Infertile	—	∞

* Controls—from start of experiment to first litter 0.5 μg. and 32.0 μg. from stopping injections to 1S litter.

weaning weights of the young. The data revealed the fact that the young did not have lower body weights at weaning, nor did the litter size show any progressive decrease throughout the series; analysis of these figures was there-

fore not attempted. In other words, litter size at birth had not been modified by the prior injection of oestrogen and there was no suggestion that a graded effect of increasing loss between birth and weaning occurred in the oestrogen-treated groups. Similarly, the percentage of young surviving to weaning in these units was not significantly different from the control group. Furthermore, it could not be concluded that the loss of whole litters was significantly more frequent in the treated groups.

The small number of young normally born in a litter and the lengthy gestation period of the guinea pig made adequate statistical treatment impossible even in this long-term experiment. Unfortunately, too, stillborn litters were often eaten before it was possible to determine the actual number of young born, which hindered the accurate compilation of breeding records. It was noted, however, that of the few young born to oestrogen-treated mothers and kept for further breeding purposes, none gave any evidence of anatomical or functional derangements and no inter-sexes were observed amongst the animals killed at weaning.

(b) *Feeding of Subterranean Clover*

(i) *Vaginal Patency.*—The figures in Table 6 indicate that a diet of oestrogenically potent subterranean clover had no effect on the duration of the oestrus phase, i.e. the time for which the vagina remained patent, or on the total length of the sex cycle. Similarly, when a normal diet was substituted for the clover ration, no irregularities or changes were evident, either in the determinations of vaginal patency or in the nature of the vaginal smears.

Table 6

EFFECT OF A SUBTERRANEAN CLOVER DIET ON THE SEX CYCLE OF FEMALE GUINEA PIGS

	Two Gestation Periods or 6 Months (10 animals/dosage level)			Two Gestation Periods or 6 Months (Five animals/dosage level)		
Treatment	Mean Time Vagina Open (oestrus) (days)	Mean Time Vagina Closed (days)	Mean Total Length Cycle (days)	Mean Time Vagina Open (oestrus) (days)	Mean Time Vagina Closed (days)	Mean Total Length Cycle (days)
Normal diet	2·9	17·6	20·5	2·7	17·0	19·7
Subterranean clover/30 g./day	3·0	16·4	19·4	2·6	11·9	14·5

Clover was withdrawn after the first two gestation periods or 6 months.

It is assumed, therefore, that the oestrogenic stimulus supplied by the clover had not affected the normal cyclic occurrence of oestrus in the female guinea pig.

(ii) *Histological Examination and Weight of the Reproductive Organs.*—The ovaries of guinea pigs receiving a subterranean clover diet revealed a

normal picture of functional activity. Genesis, development, and maturation of the follicles were apparently unaffected by treatment, and the presence of corpora lutea implied that ovulation had occurred in the usual manner. The organs were in every essential similar to those of the control animals consuming normal rations. Naturally, the change from a clover to a non-clover diet had no effect on the ovarian condition.

The accessory reproductive organs reacted to the oestrogenic factor present in the subterranean clover with both hyperplasia and metaplasia, the uterus and vagina being more severely affected than the fallopian tubes. Six months after transfer to ordinary rations, the lining epithelium of all glands had returned to normal, only isolated foci of hyperplastic activity appearing in the uterus and vagina.

Table 7

EFFECT OF A SUBTERRANEAN CLOVER DIET ON THE MEAN OVARIAN WEIGHT OF FEMALE GUINEA PIGS

Treatment	Animals	Mean Weight Ovaries (R+L) (mg.)
Normal diet	5	103·0 (2·0129)*
Subterranean clover 30 g./day	5	78·2 (1·8931)*
Normal diet	5	109·6 (2·0400)*
Clover withdrawn	5	62·9 (1·7988)*

* The figures in parentheses are means of logarithms into which the original data were converted for the purpose of statistical analysis. The remaining figures are geometric means (antilogarithms).

From consideration of the tabulated data (Tables 7 and 8), it was obvious that the mean ovarian weight of the guinea pigs consuming subterranean clover was significantly lower than that of the normally fed controls. However, the killing effect was masked by a large, though not significant, interaction. As in the previous experiment there was no significant difference in the average vaginal, uterine, and oviduct weights due to ingestion of potent clover or to the time of killing.

(iii) *Breeding Performance.*—A most remarkable feature of this second experiment was the rapidity with which the effects of the clover diet were felt —70 per cent. of the females failed to conceive and the remaining 30 per cent. were unable to carry their one pregnancy to full term. On the other hand, the controls showed a full 100 per cent. fertility for each of the two litters (Table 9). Despite the lack of conception the guinea pigs evinced normal oestrus behaviour and were served without trouble.

Even more striking was the immediate recovery made by the animals on discontinuance of clover feeding; as compared with the controls there was absolutely no delay in the resumption of breeding (Table 10).

As with the oestrogen-injected animals in the first experiment no significance was attached to the actual litter numbers, and, while the figures indicated that litter size at birth had been modified to some extent by the consumption of a clover diet, the small numbers made adequate statistical analysis impossible. There was no suggestion of increasing loss between birth and weaning in the records for the 1S litter of these animals, although the mean number of young per litter at weaning was lower than in the control group. The loss of whole litters was twice as great at the first parturition after cessation of clover feeding by comparison with the control females, and this occurred again in the 2S litter. While suggestive, these observations could not be interpreted by strict mathematical treatment.

TABLE 8

EFFECT OF A SUBTERRANEAN CLOVER DIET ON THE MEAN OVARIAN WEIGHT OF FEMALE GUINEA PIGS — ANALYSIS OF VARIANCE

Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F	P
Between classes	3	0·186920	0·062307	8·25	<0·001
Within classes	16	0·120861	0·00755381		
Total	19	0·307781			

The variation between classes was then broken up into three orthogonal components as follows:

	Degrees of Freedom	Mean of Squares	F	P
Treatments	1	0·162883	21·562	<0·001
Killings	1	0·005621	<1 n.s.	
Interaction	1	0·018416	2·438 n.s.	
		0·186920		

IV. DISCUSSION

There is no doubt that both injection of oestradiol dipropionate and ingestion of subterranean clover caused infertility in female guinea pigs and that in both cases the infertility was due to failure of conception rather than to failure of implantation.

The experimental observations demonstrated that oestradiol administration interfered with the normal ovulatory function of the animals as illustrated by oestrus derangements, atresia of the ovarian follicles, and lack of corpora lutea. It is postulated, therefore, that the high level of circulating oestrogen prevented production of sufficient LH by the anterior pituitary to effect ovulation and the normal termination of oestrus.

Graded doses of oestradiol produced graded effects, although the infertility produced by maximal dosage (32 μg.) was a permanent manifestation over the experimental period chosen. However, here the return of the sex cycles to a more usual length after discontinuance of injections indicated the beginning of a reversion to a functionally normal state. The transitory nature of the

Table 9

FERTILITY OF FEMALE GUINEA PIGS RECEIVING A SUBTERRANEAN CLOVER DIET

Treatment	Litter 1			Litter 2			Litter 1S (3)*			Litter 2S (4)*		
	Animals	Fertile	Fertility (%)	Animals	Fertile	Fertility (%)	Animals	Fertile	Fertility (%)	Animals	Fertile	Fertility (%)
Normal diet	10	10	100	10	10	100	5	5	100	5	5	100
Subterranean clover 30 g./day	10	3	30	10	0	0	5	5	100	5	4	80

* Litters 1S and 2S refer respectively to the first and second litter after cessation of clover feeding. Clover was withdrawn after litter 2.

sterility induced in the lower (0.5, 2.0, 8.0 μg.) oestrogen groups was paralleled by the records of regained fertility, normal vaginal patency, and the regression of the abnormal epithelial condition in the secondary sex organs. Presumably cessation of the exogenous oestrogen supply had subsequently permitted reorientation of the ovary-pituitary balance.

Table 10

DELAY IN REGAINING FERTILITY AFTER INGESTION OF SUBTERRANEAN CLOVER

Treatment	Litters	Mean Time to First Litter* (days)	Range (days)	Delay (days)
Normal diet	10	99·4	56—107	0
Clover withdrawn	5	88·6	73—114	0

* Control—From start of experiment to first litter. Clover withdrawn—from discontinuance of feeding to 1S litter.

With the clover-fed guinea pigs ovulation was not impaired nor were there any alterations in the cyclic occurrence of oestrus. The upset must therefore have taken place at some other point in the reproductive mechanism. Possibly the abnormal levels of circulating oestrogen interfered directly with ovum transport, or, alternatively, with the viability and motility of the sperms. The

immediate return of these females to reproductive life was surprising though irrefutable. Either only a small, though cumulatively effective, amount of hormonal activity was present in the clover, or the quantity was very great but quickly detoxicated and excreted.

It is tempting to compare the two experiments too closely and to place the amount of oestrogen ingested as clover in relation to an approximate level of oestrogen injected as oestradiol. This could be done by utilizing assay figures available, but it must be remembered that preparation of clover extracts suitable for injection and assay requires extreme measures of hydrolysis. Further, the methods of administration differed in the two cases. Nevertheless, as a point of interest it may be mentioned that the animals consuming clover received an average total of 595 μg. oestradiol equivalent each (100 g. clover $\equiv$ 14 μg. oestradiol equivalent), which amount lies within the range 2 μg. (total 238 μg.) to 8 μg. (total 952 μg.) oestradiol dipropionate injected.

Some attempt must be made to link these findings to the problem of sheep infertility in the field, despite the fact that this involves the dubious practice of arguing from one species to another. With ewes gràzing subterranean clover the massive intake of oestrogenic material might be thought to impair the gonadotrophic function of the pituitary gland, but the reported normal ovulation makes this improbable. It may be that the mechanism of sperm and ovum transport is permanently damaged. However, the extraordinary permanency of the infertility in ewes remains, at the moment, an unsolved problem.

V. Acknowledgments

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THE MODE OF ACTION OF PHENOTHIAZINE AS AN ANTHELMINTIC

II. PHENOTHIAZINE IN THE INTESTINAL FLUID AND NEMATODE PARASITES OF TREATED ANIMALS

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Summary

Methods are described for the identification of phenothiazine and its oxidation products by paper chromatography and by microionophoresis with the use of ^{35}S-labelled phenothiazine.

Extracts of the intestinal fluid from rats and chickens dosed with the drug were analysed for the presence of phenothiazine and its derivatives by these techniques. Only phenothiazine was detected in the intestinal fluid of either host but it appeared to be attached to a fatty substance from which it could not be separated by acid or alkaline hydrolysis. This phenothiazine-fat complex was not formed under anaerobic conditions *in vitro*; again, no oxidation products of the drug were detected but only phenothiazine was identified.

When *Ascaridia galli* was exposed to radioactive phenothiazine *in vivo*, 10 per cent. of the ^{35}S recovered was found in the acid-soluble fraction of the tissues and 68 per cent. in the alcohol-soluble fraction.

The parasites also appeared to contain the fatty complex of phenothiazine, and oxidation products of the drug could not be detected. The phenothiazine complex found in extracts of the gut fluid of the chicken penetrated *in vitro* the tissues of *Ascaridia galli* at a slower rate than did a suspension of the pure drug.

The results of the investigation indicate that phenothiazine itself, not its oxidation derivatives, is the anthelmintic agent.

I. INTRODUCTION

When phenothiazine was given to rats infested with *Nippostrongylus muris* and to chickens infested with *Ascaridia galli,* phenothiazine and its sulphur-containing derivatives, if such compounds were formed, appeared in the tissues of both parasites in similar amounts and remained in the parasites for long periods (Lazarus and Rogers 1950, 1951). However, as the result of the treatment *Ascaridia galli* was expelled from the host whereas *Nippostrongylus muris* appeared to be unaffected by the drug. The difference in action of the drug on the two parasites might be explained if toxic derivatives of phenothiazine were formed in the gut contents of the chicken or in the tissues of *Ascaridia galli* but not in the intestinal contents of the rat or its parasite. Another explanation of the differential toxicity of the drug to the two parasites might be attributed to differences in the metabolism of the para-

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sites. It is known, for instance, that the metabolism of *Nippostrongylus muris* is predominantly aerobic *in vivo* whereas that of *Ascaridia galli* is probably anaerobic (Rogers 1949).

The investigation described in this paper was carried out to examine the first hypothesis. Attempts have therefore been made to discover whether appreciable amounts of phenothiazine derivatives are formed in the gut contents of host animals treated with the drug and in their parasites, and whether the nature of the derivatives formed differs in different animals.

II. Methods

(*a*) *Biological Materials*

Ascaridia galli was obtained from experimentally infested chickens. After collection, the parasites were washed repeatedly in normal saline until free from debris and contaminating radioactive material.

Chickens that had been fasted overnight were dosed with phenothiazine directly into the crop. Rats were also fasted overnight and lightly anaesthetized with ether before phenothiazine was administered through soft catheter tubing directly into the stomach.

Phenothiazine labelled with ^{35}S was always diluted with suitable amounts of carrier before use and traces of wetting agent were added to facilitate suspension in water.

The intestinal fluid, referred to later, was obtained immediately after the animal was killed; the gut was ligated, the contents were collected and centrifuged. The supernatant fluid was used.

(*b*) *Preparation of Phenothiazine and its Derivatives*

Radioactive phenothiazine was synthesized from elemental ^{35}S and purified according to Lazarus and Rogers (1951). Large quantities of the unlabelled drug were purified from the commercial product by the method of Baker and Brickman (1945). The melting point of the pure phenothiazine obtained from both methods was 181-183°C.

Phenothiazone-3 was prepared by the method of Pummerer and Gassner (1913) and further purified by recrystallization, once from alcohol and once from water, m.p. 160-161°C.

The thionol and phenothiazine-5-oxide were gifts from Dr. Floyd De Eds, U.S. Department of Agriculture.

(*c*) *Separation of Phenothiazine and its Derivatives by Paper Chromatography*

A mixture containing 50 μg. each of phenothiazine and the derivatives, dissolved in 10 μl. of hot methanol, was applied to Whatman No. 1 special chromatography paper and the action of solvents was examined by the 1-dimensional ascending chromatographic technique of Williams and Kirby (1948). The R_F values of the substances in some of the many solvents tried are given in Table 1. It was found that a minimum of 10-20 μg. of phenothiazine, thionol, and phenothiazone-3 and 20-30 μg. of phenothiazine-5-oxide could be detected by the chromatographic method.

A good separation was effected by means of a 2-dimensional chromatogram with an acetone and water mixture in the proportions of 3 to 1, followed by absolute methanol. In each case the developing solvent was also used to saturate the atmosphere. A diagram of the chromatogram is given in Figure 1.

A 1 per cent. alcoholic solution of iodine sprayed on to the paper showed up the phenothiazine-5-oxide as a yellowish-brown spot which faded quickly, and the green, red, and blue colours of phenothiazine, phenothiazone-3, and thionol respectively were intensified by this reagent. However, the positions of the spots on the chromatogram could usually be detected without spraying.

Table 1

R_F VALUES OF PHENOTHIAZINE DERIVATIVES IN SEVERAL SOLVENTS

Compound	Acetone +Water 3 : 1	Absolute Methanol	Acetone +Methanol +Water 3 : 2 : 1	Dioxane +Water 2 : 1	Benzene +Methanol 3 : 1	Methyl Ethyl Ketone+Water 1 : 1	Butanol +Water 1 : 1
Phenothiazine	0·97	0·69	0·94	0·98	0·98	0·99	0·89
Phenothiazone-3	0·83	0·59	0·80	0·90	0·97	0·96	0·88
Thionol	0·66	0·45	0·58	0·82	0·43	0·94	0·70
Phenothiazine-5-oxide	0·85	0·68	0·83	0·91	0·75	0·94	0·89

(*d*) *Identification of Phenothiazine and its Derivatives Associated with Biological Materials*

Chromatograms of extracts of parasites and intestinal fluids of the host animals were prepared. The spots, cut from the chromatograms that had not been sprayed with iodine, were extracted with methanol and the extracts examined in a Beckman spectrophotometer, model D.U.

When the drug labelled with ^{35}S was used, radio-autographs were made of the chromatograms, or the distribution of activity in the chromatographed material was measured by the method of Taurog, Tong, and Chaikoff (1949) of passing the filter paper underneath the tube of a Geiger-Müller counter and determining the counts per minute at regular intervals along the strip.

Chromatograms were examined for the presence of proteins by the method of Wynn and Rogers (1950), tests for carbohydrates were made according to Trevelyan, Procter, and Harrison (1950) and a 2 per cent. aqueous solution of osmic acid was applied to detect the presence of fats.

(*e*) *Spectrophotometric Analysis*

The pure substances phenothiazine, thionol, phenothiazone-3, and phenothiazine-5-oxide, dissolved in methanol, were examined by spectrophotometry; their spectra agreed with those obtained by Houston, Kester, and De Eds

(1949*a*, 1949*b*). However, this method of analysis was found to be too insensitive for the identification of the oxidation products of phenothiazine in the amounts likely to be present in the intestine.

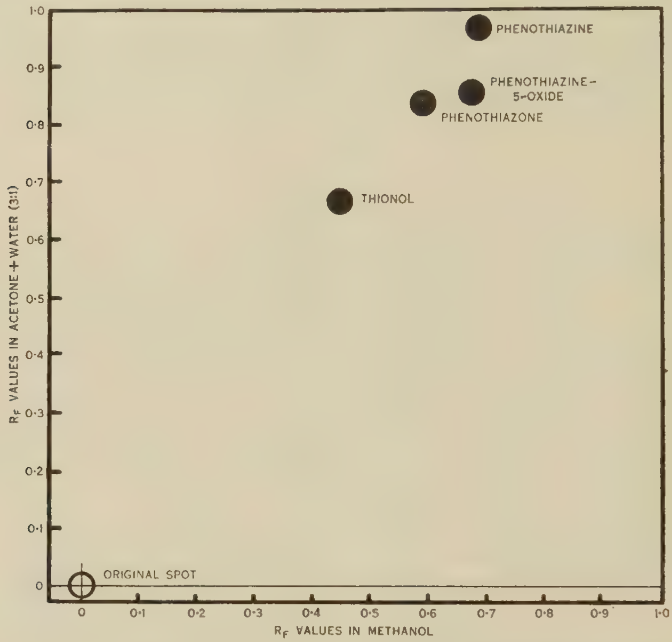

Fig. 1.—Two-dimensional chromatogram of phenothiazine and derivatives, using acetone plus water (3 : 1) followed by absolute methanol as solvents.

(*f*) *Microionophoresis*

Phenothiazine and its derivatives, in the pure state and in association with biological materials, were subjected to treatment by the microionophoretic technique of Durrum (1950), with a slightly modified apparatus. The solution to be analysed, 0.1 ml. containing 50 μg. each of phenothiazine and the derivatives, was applied as a circle of about 6 mm. diameter at the centre of a strip of Whatman No. 1 filter paper, 37 by 1 cm. When dry, the strip was saturated with the electrolyte, 0.2M phosphate buffer, pH 6.0, by Durrum's technique of hanging the paper over a glass rod and applying the electrolyte on either side of the test spot with Pasteur pipettes. After it had drained well, the strip was placed between two slabs of plate glass and set up as shown in Figure 2. A potential, usually of about 600 V., obtained from dry cells, was applied across the carbon electrodes in series with a milliammeter and a rheostat. The current usually varied from 2 to 6 mA. At the end of 3-6 hours the strip was removed, dried, and sprayed with a colour reagent, either iodine or dilute hydrochloric acid; the latter converts phenothiazine compounds into pink thionium derivatives.

It was found that thionol migrated towards the anode, phenothiazone-3 and phenothiazine-5-oxide moved towards the cathode, and phenothiazine itself was electrically neutral.

The migration of radioactive compounds was followed by measuring the activity of strips 2 mm. wide under the tube of a Geiger-Müller counter.

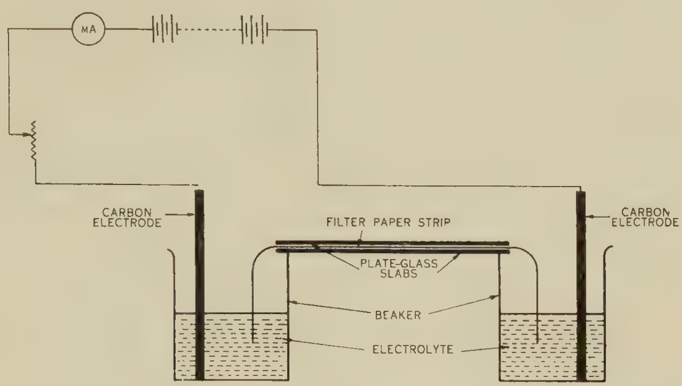

Fig. 2.—Apparatus used for separation of phenothiazine derivatives by microionophoresis. For explanation see text.

III. Procedure and Results

(a) *Phenothiazine in the Intestinal Fluid of the Host*

Groups of four normal rats, weighing approximately 100 g. each, were fasted overnight and then dosed, 1 g./kg., with phenothiazine. Each dose was given in 2 ml. of water, containing small amounts of an anionic detergent. In tracer experiments, 1-2 μc. of ^{35}S-labelled phenothiazine was included in the dose.

After 1½ hr. the rats were killed, the small intestines ligated, and the contents of the small intestines collected, pooled, and centrifuged; the supernatant fluid was extracted with 30 ml. of oxygen-free hot methanol or benzene and the extract was then concentrated *in vacuo*. All operations were carried out in an atmosphere of nitrogen.

Chickens were also fasted overnight and dosed with appropriate amounts of the drug, either in the non-radioactive form or containing 2-4 μc. of ^{35}S-labelled material. The birds were killed after 6 hr. and the intestinal fluid was collected and extracted in the same way as from the rats. The fluid from one chicken was sufficient for one experiment.

Intestinal fluid extracts from both rats and chickens behaved in a similar manner on chromatograms. When developed with the acetone plus water solvent, the material on the chromatogram showed the green colour of phenothiazine but trailed down the paper as if some other substance were interfering. When developed further with absolute methanol, the substance that trailed in the acetone plus water solvent gave R_F values to be expected from phenothiazine.

It should be stated here that sometimes the presence of phenothiazone also was noted on the chromatograms. However, at no time was phenothiazone observed in ionophoresis experiments, so its occasional appearance on chromatograms might have been due to unavoidable aerial oxidation.

Extracts of blood and bile from dosed chickens contained phenothiazine, which trailed in the acetone plus water solvent. In some experiments phenothiazone and thionol were identified in bile extracts.

The material associated with the phenothiazine on the chromatogram was negative to tests for proteins and carbohydrates but positive to tests for fats. No trailing was observed in chromatograms of extracts of intestinal fluid that had been incubated anaerobically *in vitro* with phenothiazine. Thus it appeared that the phenothiazine complex formed was peculiar to conditions *in vivo*. A comparison of chromatograms of *in vivo* and *in vitro* extracts is shown in Figure 3.

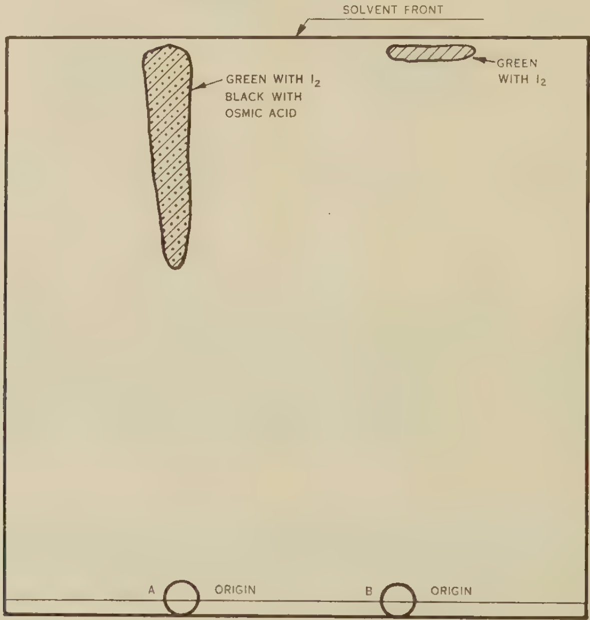

Fig. 3.—Typical chromatogram of an extract of intestinal fluid plus phenothiazine *in vivo, A*, and *in vitro, B*.

Attempts to hydrolyse the phenothiazine-fat complex with boiling 1N HCl for 2-3 hr. and with boiling 5N NaOH for 4 hr. failed.

Figure 4 gives a typical curve for the distribution of radioactivity in the chromatograms of intestinal fluid extracts from chickens dosed with the ^{35}S-labelled drug. A previous dose of peanut oil given to chickens to increase the fat content in the small intestine did not increase the trailing effect.

Gut fluid extracts were examined by microionophoresis and the results were compared with the ionophoretic behaviour of pure phenothiazine compounds. No migration to anode or cathode by extracts of intestinal fluid was observed, so it appeared that phenothiazine was, indeed, the only compound present.

(b) *Phenothiazine in the Parasites*

Unfortunately the infestations of *Nippostrongylus muris* in the rats gave insufficient material for the examination of phenothiazine compounds in the parasites, therefore these experiments were confined to *Ascaridia galli* from chickens.

The worms were collected 6-7 hr. after the host had been dosed with ^{35}S-labelled phenothiazine. The parasites were extracted with alcohol, which had been boiled to remove oxygen, for a period of 2-4 hr. in a Soxhlet apparatus. The alcoholic extract, concentrated *in vacuo*, was then chromatographed in acetone and water. It trailed on the paper in a similar manner to the extract from the host's intestinal fluid. However, the distribution of radioactivity in the chromatographed material was reversed (Fig. 5). This may have been due to the fact that the intestinal fluid extract was prepared in a different manner from the extract of the parasites, but in fact no adequate explanation has yet been found.

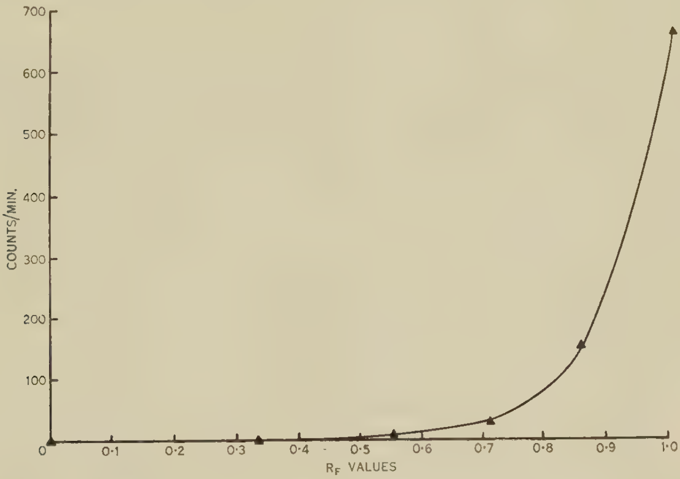

Fig. 4.—Distribution of radioactivity in chromatograms of extracts of intestinal fluid taken from chickens dosed with ^{35}S-labelled phenothiazine. The developing solvent was acetone plus water (3 : 1).

Ionophoresis of alcoholic extracts of the worms again indicated the presence of phenothiazine only.

A comparison was made of the rates of penetration *in vitro* into *Ascaridia galli* of the phenothiazine complex found in intestinal fluid extracts and of pure phenothiazine. Worms were collected from an undosed chicken and thoroughly washed in saline. The intestinal fluid of a chicken dosed with the ^{35}S-labelled drug, containing wetting agent, was extracted with benzene, concentrated *in vacuo* to 0.5 ml., and made up to 4 ml. with buffered saline (flask *A*). A suspension of radioactive phenothiazine in saline, containing 0.05M phosphate buffer at pH 6.0 and a trace of wetting agent, was made up so that 4 ml. of the suspension (flask *B*) was equal in radioactivity to the 4 ml. of the intestinal

fluid extract. One group of parasites was incubated in flask *A* and one in flask *B* for 2 hr. at 37°C. Then the parasites were washed repeatedly in saline, dried between filter papers, and prepared for assay of radioactivity by the method of Lazarus and Rogers (1951). The phenothiazine in the intestinal fluid extract was taken up at a slower rate than the phenothiazine in the suspension (Table 2).

TABLE 2

UPTAKE OF PHENOTHIAZINE BY *ASCARIDIA GALLI* AFTER EXPOSURE TO THE DRUG *IN VITRO* FOR 2 HR.

Medium	Phenothiazine Uptake (μg./g. wet wt.)
Buffered saline plus intestinal fluid extract	19, 13, 19, 17 (17)
Buffered saline plus wetting agent	19, 31, 26, 24 (25)

The figures in brackets show the mean value of the phenothiazine uptake; four experiments were carried out with each medium.

The tissues of *Ascaridia galli,* exposed to ^{35}S-labelled phenothiazine *in vivo,* were extracted by the following procedure in order to determine in what fraction the ^{35}S was concentrated.

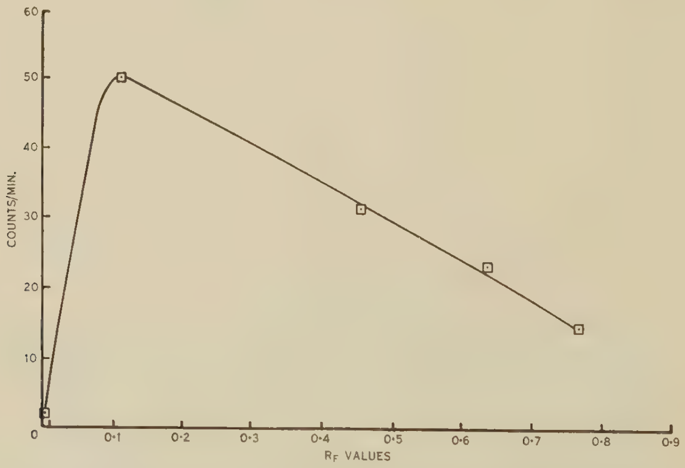

Fig. 5.—Distribution of radioactivity in chromatograms of alcoholic extracts of *Ascaridia galli* dosed *in vivo* with ^{35}S-labelled phenothiazine. The developing solvent was acetone plus water (3 : 1).

The worms were collected, washed well in saline and ground with cold 7.5 per cent. trichloracetic acid. Aliquots of the brei were assayed for total ^{35}S content (Lazarus and Rogers 1951). The remainder was centrifuged at 3,000 r.p.m. (*c.* 1300g) and aliquots of the supernatant were used to determine the acid-soluble ^{35}S. The residue was washed once with cold 8.5 per cent. trichloracetic acid, dried in a desiccator, and the supernatant discarded. The dried

residue was extracted with alcohol and alcohol plus ether (3 to 1), the extracts were combined, and the alcohol-soluble ^{35}S estimated. The amount of ^{35}S in the final residue was also estimated. From Table 3 it can be seen that most of the ^{35}S was contained in the alcohol-soluble fraction of the tissues of *Ascaridia galli.*

TABLE 3

DISTRIBUTION OF ^{35}S IN THE TISSUES OF *ASCARIDIA GALLI* DOSED WITH RADIO-ACTIVE PHENOTHIAZINE *IN VIVO*

Fraction	Percentage ^{35}S Recovered
Acid-soluble	10
Alcohol-soluble	68
Residue	22

IV. Discussion

The immediate problem involved in the study of the mode of action of phenothiazine as an anthelmintic is the determination of the form in which the drug is active. Very large doses of the drug are required to obtain efficiency; the drug, though it enters the tissues of *Nippostrongylus muris*, has no anthelmintic action on this species (Lazarus and Rogers 1951). Further, the efficiency of phenothiazine against a species normally affected by the drug is variable and no satisfactory explanation of this variability has been given. It is possible, therefore, that phenothiazine itself is not the active agent and that the activity is due to other substances, either present in the drug as contaminants or formed as derivatives of phenothiazine within the host. The first suggestion is evidently incorrect because highly purified phenothiazine given with a suitable detergent is as active as the ordinary commercial product. The alternative explanation, however, requires examination.

The biological activity of phenothiazine and its derivatives against enzyme systems of host animals has been studied by Collier (1940) who found that mammalian catalase and cytochrome oxidase were not affected by phenothiazine but were strongly inhibited by leucophenothiazone, leucothionol, and thionol. Collier and Allen (1942) also reported the inhibition of cholinesterase by phenothiazone.

Field tests by Gordon and Lipson (1940) showed that phenothiazone had no anthelmintic effect on parasites in sheep and that thionol was ineffective against *Haemonchus contortus* and *Trichostrongylus* spp. Guilhon (1947), who worked with pigeons, also found that thionol was inactive *in vivo* against *Ascaridia colombae.* However, De Eds and Thomas (1941) examined the effect of thionol *in vitro* on *Ascaris lumbricoides* and claimed that thionol was a more effective poison than phenothiazine.

Whitten (1948) found that the activity of phenothiazine-5-oxide against the parasites of sheep was comparable with that of phenothiazine. However, as Whitten pointed out, only a small proportion of phenothiazine administered

to an animal is converted to the sulphoxide. Therefore, if the latter were responsible for the anthelmintic effect, a higher efficiency would be expected when the pure substance was given. No information is available concerning the stability of phenothiazine-5-oxide in the alimentary tract, especially as regards the possibility of its reduction to phenothiazine. Guilhon (1948) found the sulphoxide to be ineffective against *Ascaridia colombae* in pigeons.

Harpur, Swales, and Denstedt (1950) and Harpur, Denstedt, and Swales (1950) showed that after oral administration, the absorption of phenothiazine from the intestine was not dependent on oxidation to phenothiazone in the rumen. Indeed, the presence of phenothiazine in blood draining the rumen indicated that the drug was absorbed from it in the unoxidized state. The presence of leucophenothiazone was also observed in the peripheral circulation of lambs dosed with phenothiazine. No direct information on the anthelmintic activity of phenothiazine was brought forward by these authors but they are of the opinion that it is "attributable to the unabsorbed, but not necessarily unaltered fraction of the drug."

The results of the present investigation suggest that phenothiazine is the major component found in the intestinal contents and in the parasites treated with the drug. Other derivatives, especially those containing sulphur, were not detected and could not have been present in appreciable amounts. The conditions prevailing in the intestine of the host are unlikely to favour the formation of oxidation products of phenothiazine and these conditions were duplicated as far as possible during *in vitro* experiments.

Although it appears that conditions in the gut contents are such that the "chemical" nature of phenothiazine is not markedly affected, it is still possible that the physical state of the compound is important in relation to its anthelmintic activity. So far, little information concerning this matter is available. Phenothiazine, under some circumstances, can exist as resonance-stabilized free radicals (Murphy, Ravner, and Smith 1950). The significance of this mechanism in relation to biological activity is unknown.

The present work has shown that phenothiazine, both in the host's intestine and in the worms, appears to be attached to a fatty substance. It was thought that the nematode cuticle, which is largely albuminoid (Chitwood 1936), probably with a fatty surface layer (Trim 1949), might be more rapidly penetrated by such a fatty complex. However, experiments indicate that the presence of the fat does not increase the rate of uptake of the drug by the parasites.

It is clear from this investigation that further study is required concerning the biological action of phenothiazine itself in the tissues of parasites. Nematodes poisoned with phenothiazine are not unduly affected by it in aerobic environments (Lazarus and Rogers 1951); therefore the biological lesion caused by phenothiazine may be found among the mechanisms by which parasites obtain energy from anaerobic sources.

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CORRIGENDUM

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Page 380, last paragraph but one: The three lines of this paragraph should be replaced by the following:

There is some dispute as to the actual proportions of the volatile fatty acids produced in ruminal digestion (Gray and Pilgrim 1950; Kiddle, Marshall, and Phillipson 1951), but if it is taken, as appears reasonable, that:

(i) 70 per cent. of the energy absorbed as volatile fatty acids is provided by acetic and butyric acids,

(ii) The volatile fatty acids provide 80 per cent. of the metabolizable energy of the feed, digestible carbohydrates providing nil, and amino acids and fat providing say 20 per cent., and

(iii) Acetic and butyric acids have specific dynamic actions of 50-70 per cent.,
then the resultant figure of 28-39 per cent. for heat increment due to the metabolism of acetic and butyric acids from the feed could account for a major proportion of the total heat increment, reported as 30-60 per cent. of the metabolizable energy. Additional sources of heat increment would be, of course, the actual heat of fermentation, calculated by Marston (1948) to provide 15 per cent. of it, and the energy costs of metabolism of propionic acid, amino acids, and fat. The present theory thus appears to be quantitatively adequate.

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